The role of mast cells in prostate cancer initiation and recurrence

By Heidi Anne Hempel

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Abstract

Mast cells are known for their role in allergic reactions via the IgE-antigen complex and degranulation. Mast cells are resident in most healthy tissues, including the prostate. In addition, mast cells are present in higher densities in prostate cancer with lower Gleason scores than in higher Gleason scores, suggesting potential interactions between mast cells and cancer cells. Consequently, a number of studies have explored the relationship between mast cell density and prostate cancer progression, albeit with varied results. Contradictions in the literature on whether mast cell density is associated with a good or poor prognosis may be due to variability in sampling or staining methods, or may represent true phenotypic differences between mast cells inside and outside the tumor.

In this dissertation, we sought to explore the relationship between mast cells and prostate cancer progression and race disparities, as well as explore phenotypic differences between mast cells located inside or outside the tumor. The association between mast cells and prostate cancer progression was accomplished via a double immunohistochemistry (IHC) stain for mast cell tryptase and epithelial Cytokeratin-8 (Ck8) on a tissue microarray (TMA) set designed to determine associations with biochemical recurrence (PSA progression) after prostatectomy. Total mast cell number, mast cell density (mast cell number/tissue area), and mast cell number per epithelial or stromal area was assessed via a novel Aperio-based image analysis software termed PIP. Our findings indicate that high intratumoral mast cell density is associated with a lack of PSA recurrence, while high extratumoral mast cell density was correlated with a higher likelihood of PSA progression. Analysis of a role for mast cells in prostate cancer racial disparities was accomplished via similar staining of several matched

African American (AA) and Caucasian American (CA) prostate cancer TMAs. Results indicated that tumors from AA men with lower grade tumors (Gleason grade ≤ 3+4=7) had significantly lower numbers of mast cells than CA men matched on age, grade, and stage. Furthermore, intratumoral mast cell numbers in tumors from AA men had a similar relationship with PSA progression as in the PSA progression TMA study, and we additionally observed an association between lower intratumoral mast cells and increased rates of metastasis in both CA and AA men.

Finally, using a number of techniques, the mast cell phenotype inside and outside the tumor was explored. Mast cell subtype was determined in AA and CA prostate tissues using double immunofluorescence (IF) staining for mast cell tryptase and chymase. Mast cells located in the tumor and benign regions and their immediate microenvironment were captured by laser capture microdissection (LCM) followed by RNA sequencing (RNAseq). These experiments revealed numerous differentially expressed genes between intratumoral and extratumoral mast cells. To validate and further expand on these findings, C-kit variant 1, CXCR4, and TFE3, were evaluated using quantitative PCR and *in situ* hybridization techniques.

In addition, we employed two animal models to elucidate the role of mast cells in tumor invasion in the rodent prostate. In a rat model whereby pre-invasive neoplastic lesions are induced by the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), mast cell numbers were quantified in the ventral prostatic lobes of rats that had been treated with PhIP and then aged another 32 weeks. This allowed us to elucidate that mast cell number was increased long after exposure to PhIP, specifically in the ventral prostate. We also crossed a mast cell knock-out mouse, known as the Wsh mouse, with a well-known prostate cancer mouse model induced by overexpression of the oncogene cMYC (HiMyc), in

order to determine if the absence of extratumoral mast cells would result in lower invasive tumor area in the mouse prostates. These results confirmed our hypothesis by demonstrating that mast cell knock-out mice displayed a lower overall invasive cancer area. In addition, we found that the dorsal-lateral lobe of the prostate both contained the highest number of mast cells of all the lobes, as well as the highest invasive tumor area. The results from both rodent models suggest a role for mast cells in the lobe specificity of these rodent models, as well as a potential role for mast cells in early tumor development and invasion.

Advisor/Reader: Karen Sandell Sfanos, PhD

Reader: Christopher Heaphy, PhD

Committee Members: Noel Rose, PhD (chair)

Donald Coffey, PhD

Angelo De Marzo, MD, PhD

Charles Drake, MD, PhD

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I. INTRODUCTION: MAST CELLS IN PROSTATE CANCER

Mast cells are best known for their role in allergic reactions, where IgE-antigen complex (IgE-Ag) binding to the mast cell receptor FceRI stimulates degranulation, releasing effector molecules such as histamine, serotonin, leukotrienes, and proteoglycans [1,2]. However, mast cells have also been shown to play significant roles in defending against parasites, controlling bacterial infection, and producing immunoregulatory cytokines [1,3-6]. What makes mast cells particularly interesting in cancer biology, however, lies in the dynamic way in which these cells reside in and interact with the microenvironment in their target tissues. Mast cell functions can have potent effects on their environment that can be powerfully pro- or anti-tumorigenic, depending on the circumstances (Figure 1.1).

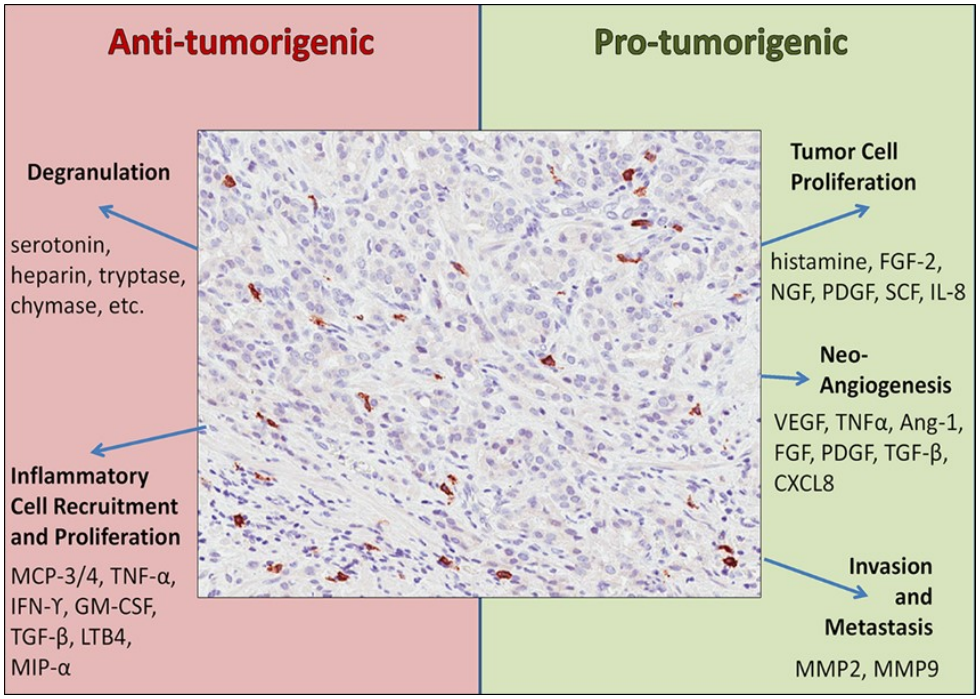


Figure 1.1 Potential biological roles for mast cells in prostate cancer. Photomicrograph shows a region of human prostatic adenocarcinoma stained for mast cells (brown staining cells) by immunohistochemistry. Original magnification, x 100

These functions are dynamic and subject to manipulation by outside forces, possibly even by the surrounding cancer cells [1,7]. As such, it is likely that mast cells play distinctive roles in different cancers types and across stages [8]. This possibility is, in fact, reflected in the literature. There have been a number of studies in numerous cancers attempting to correlate mast cell density in or around the tumor, with the results varying from associating mast cell density with good prognosis, poor prognosis, or having no association with prognosis at all, even between studies in the same tumor type [1,8-24] . In prostate cancer specifically, higher numbers of "intratumoral" mast cells have been shown to be correlated with lower Gleason grade and better prognosis [22-24], however the opposite finding has also been reported [25]. These and other studies on mast cells in human cancers were performed using a variety of techniques and without a standardized definition of "intratumoral" versus "peritumoral" mast cells. Nevertheless, the notion of mast cells having different effects in different circumstances is an attractive one, and is consistent with what is known about mast cell biology.

Mast cells, like most immune cells, originate in the bone marrow. However, mast cells travel to their targets before their final stages of development, and their final differentiation into different subtypes is dictated in large part by the microenvironment of their resident tissues [1,7]. The two main subtypes of mast cells are commonly known as MC_{TC}, which express chymase, tryptase, carboxypeptidase, and cathepsin G and are usually found in mucosa, and MC_T, which express mainly tryptase, and are usually localized to connective

tissues [3,26,27]. These two subtypes also differ in number, type, and content of secretory granules, as well as which stimuli to which they will respond [7]. These subtypes are not exhaustive, however, as mast cells have been discovered with additional phenotypes [7]. In addition, there is evidence to suggest that mast cell differentiation is not final, and the phenotype and mast cell protease profile can be changed based on different conditions in their microenvironment, including exposure to different cytokines, the presence of fibroblasts, and different host organ tissues [1,28,29]. As such, it is reasonable to suggest that mast cell phenotype will differ depending on the conditions, such as *in vitro* versus *in vivo*, and in different cancers and cancer stages.

The best known method of mast cell effector function is via degranulation, which is the release of the previously synthesized contents of intracellular granules in response to IgE-Ag binding to the mast cell receptor. These contents include effector molecules such as serotonin, histamine, heparin, tryptase, and chymase, among others, many of which with pro-inflammatory, anti-tumorigenic properties (Figure 1.1). However, there is also evidence for a method for "piecemeal degranulation" of mast cells, allowing for the selective release of cytokines without the release of the entire secretory granule [30]. This alternative activation model helps to fuel the mechanistic side of the argument for the pro-tumorigenic potential of mast cells, as it provides the potential for the selective release of pro-tumorigenic cytokines such as IL-1 and IL-6 [31] and effector molecules such as MMP9 in the absence of anti-tumorigenic mast cell granule components. The mast cell subtype also comes into play here, as different subtypes will have distinctive effector molecules available to them, and thus can have different effects on the tumor upon degranulation, piecemeal or otherwise. In addition, mast cells are also potent producers of immune modulating cytokines, chemokines,

angiogenic factors, and proteases, all of which can cause significant changes in the tumor microenvironment [27].

Mast cells are known to produce many different angiogenic molecules, including VEGF, histamine, TNFa, and Ang-1, and thus have been suspected for some time to play a significant role in tumor angiogenesis, and possibly even in the angiogenic switch [32]. There have been several studies supporting this idea, including a transplantation multiple myeloma mouse model, in which it was found that transplanting mast cells with plasmacytoma tumors resulted in significantly higher vascularization [33]. In addition, one study in a squamous carcinoma mouse model demonstrated a significant decrease in pre-malignant angiogenesis in mast cell deficient mice [34]. Evidence in humans for a role for mast cells in tumor angiogenesis is also mounting, such as in one study correlating melanoma progression with mast cell density and simultaneously increased vascular density [15]. However, some have also suggested a role for mast cells in suppressing angiogenesis by providing receptors that can "soak up" angiogenic factors [7]. In addition, there are studies correlating mast cell density and cancer progression without finding a correlation with angiogenesis, suggesting that mast cells may also have a role in tumor promotion aside from blood vessel development [13,14]. In human prostate cancer, mast cell densities have not been correlated to neovascularization, however in an orthotopic rat model, implantation of AT-1 tumor cells resulted in peritumoral recruitment of mast cells and an increase in peritumoral vascular density [22]. Furthermore, castration was found to result in mast cell recruitment to the prostate both in men and in the AT-1 tumor model and the Dunning rat model, and this correlated to an increase in vascular density in the Dunning model [22].

In addition to the possible roles for mast cells in angiogenesis, the role of mast cells in tissue remodeling is of particular interest in tumor promotion and includes a possible connection in prostate cancer. The idea that the extracellular matrix (ECM) plays a significant role in tumor promotion has gained importance in recent years [35-37]. Mast cells produce potent proteases, including chymase, tryptase, collagenases, MMP9 and other gelatinases, and cysteinyl cathepsins. As such, research into the role of mast cells in ECM modulation and tumor invasion is also gaining ground [27]. One study in prostate cancer explored the role of mast cell MMP9 in early prostate tumor progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, arguing that lower grade prostate tumors would need mast cell-derived MMP9 for invasion, since well differentiated prostate tumor cells do not produce MMP9 [38]. The results did in fact suggest that mast cell MMP9 was necessary for early tumor invasion in mice. Immunohistochemistry (IHC) studies in human prostate cancer tissues showed a positive correlation between increased mast cell density, MMP9 production confined almost entirely to tumor-infiltrating mast cells, and well differentiated tumors – supporting the observations observed in the TRAMP mouse studies [38]. Whether mast cell-derived MMP9 plays a role in driving early invasion of human prostate cancer is yet to be elucidated.

In addition to the direct effects mast cells might have on cancer cells and their microenvironment, mast cells may also affect cancer through their roles as potent immune modulators. Mast cells are capable of both suppressing and promoting inflammatory responses, depending on the circumstances [1]. In fact, mast cells are known to produce a number of cytokines and chemokines capable of recruiting, activating, suppressing, and driving the differentiation of both innate and adaptive immune cells, including neutrophils,

basophils, macrophages, lymphocytes (such as B cells, T_H2 T cells, and T_{reg} cells), and natural killer cells. As such, mast cells are also capable of anti- or pro-tumorigenic effects through the suppression or activation of the inflammatory response, and could also have a significant effect on other inflammatory cells in different cancers.

Studies both *in vivo* in mice and *in vitro* using mouse and human cells have demonstrated that mast cells directly influence cancer cells, with one *in vivo* study showing decreased mitotic index and increased apoptosis in intestinal polyps, and two *in vitro* studies showing increased proliferation and invasion upon treatment with mast cell conditioned medium [27,39-41]. This activity of mast cells has been theorized to be due to the production of tryptase, which is reported to promote proliferation of colon cancer cells, fibroblasts, and other cell types [42-47]. Mast cell histamine has also been suggested to play a role in tumor cell proliferation, however the literature is not in agreement on whether it promotes or suppresses proliferation [7]. In contrast, mast cell effector molecules such as IL-4 and TNFα could result in tumor cell death [5,48].

As mast cells and cancer cells evolve in the same microenvironment, the interactions between these cell types could change significantly. With the myriad of mast cell effector molecules, there is a delicate balance between the anti- and pro-tumorigenic capabilities of mast cells that can conceivably change dramatically with time, even within the same tumor. It is also possible that even if mast cells cannot serve as an independent prognostic factor for all cancers, they might be an indicator of cancer aggressiveness and invasiveness [1]. In addition, it is important that this ever-evolving relationship with cancer be better understood before any mast cell-targeted therapies are attempted. Since mast cells may have the potential to be pro-tumorigenic or anti-tumorigenic depending on the microenvironment, even in the

same cancer, certain therapies may be beneficial at certain stages of cancer, and detrimental in others [8,38]. As such, mast cells have great potential in cancer research, and the many possible roles of mast cells in prostate cancer continue to be an important area of study.

II. LOW INTRATUMORAL MAST CELLS ARE ASSOCIATED WITH PROSTATE CANCER RECURRENCE

2.1 Abstract

Mast cells are of interest in prostate cancer because they possess both pro- and anti-tumorigenic properties and are present in the tumor microenvironment. We studied the association of mast cell count and densities with prostate cancer recurrence using tissue microarrays (TMAs) for 462 men who recurred (cases) and 462 controls that were matched to the cases nested in a cohort of radical prostatectomy patients. Dual-immunostaining for mast cell tryptase and epithelial cytokeratin-8 and whole slide image analysis were used to assess total mast cell number, mast cell density (mast cell number/tissue area), and mast cell number per epithelial or stromal area in TMA spots containing tumor (up to 4 per man). We used conditional logistic regression to estimate the odds ratio (OR) and 95% confidence interval of recurrence for the mean, minimum, and maximum mast cell parameters in tumor tissue among each man's TMA spots. After taking into account matching factors of age, race, Gleason sum, and pathologic stage, higher minimum mast cell density in the tumor (comparing highest to lowest quartiles: OR=0.58, 95% CI 0.40-0.86; P-trend=0.004) was

associated with a lower risk of recurrence. Patterns for mast cell number and ratio of mast cell number to epithelial or stromal area were similar to those for mast cell density. Our results suggest that intratumoral mast cells may be protective against prostate cancer recurrence and could potentially serve as a prognostic biomarker after prostatectomy.

2.2 Introduction

Inflammation is known to play a significant role in the development of many cancers, and may promote carcinogenesis and cancer progression through a variety of mechanisms including stimulating tissue remodeling and tumor-promoting changes in the extracellular matrix, loss of tissue architecture, DNA damage due to oxidative stress, promotion of angiogenesis, and direct stimulation of tumor cells via cytokines [7,49,50]. Evidence for a potential role for inflammation in prostate cancer is steadily building, as inflammation is frequently observed upon histological examination of prostate biopsies, radical prostatectomy specimens, and autopsy prostate specimens [51]. Likewise, inflammation is proposed to be a key factor in the development of a risk-factor/putative precursor lesion called proliferative inflammatory atrophy (PIA) that is frequently seen to be in transition to prostatic intraepithelial neoplasia (PIN) and at times directly to adenocarcinoma [52-54]. Multiple studies have also found significant correlations between genetic polymorphisms and aberrant expression of inflammation-related genes in prostate cancer [51,53,55]. In addition, inflammatory cells and mechanisms of immune modulation and tolerance have been suggested to play key roles in castration resistance [56-58]. However, the inflammatory cell profile in different regions or stages of prostate cancer, and the mechanisms by which this inflammation may be

contributing to progression and recurrence are not well understood [51]. There is evidence suggesting roles for both adaptive and innate immune cells, with T and B cells emerging as having roles in castration resistance and immune tolerance, and macrophages possibly having a driving role in prostate cancer progression and metastasis [56-61]. In addition to these cell types, mast cells are emerging as immune cells of particular interest in prostate cancer etiology [62].

Mast cells are of interest in the context of cancer for a variety of reasons. Mast cells are resident in many tissues throughout the body, including the prostate, and, unlike many inflammatory cells, do not undergo full differentiation until they are established in the tissue microenvironment [7,28]. This differentiation may not be final, however, and there is a possibility that mast cells within a tumor will be markedly different in their protease profile or subtype than mast cells outside the tumor [1,28,63,64]. In addition, mast cells may have the ability to change their profile as the tumor microenvironment evolves [1,28,63,64]. This potential for plasticity is relevant since mast cells are extremely diverse in their functions, and have both pro- and anti-tumorigenic properties and functions [8,63]. For instance, mast cells are highly pro-inflammatory upon degranulation, and are known to recruit neutrophils, eosinophils, macrophages, and B and T cells capable of mounting an anti-tumor immune response [8,65-67]. In contrast, mast cells are also potent producers of VEGF that could promote angiogenesis, and MMP9 that could modulate the extracellular matrix and possibly promote tumor invasion [7,8,65,68]. Mast cells have been shown to interact with T and B cells, regulatory T cells, neutrophils, natural killer (NK) cells, dendritic cells, and macrophages, and produce cytokines that can both modulate the immune system (e.g. IL-17) and directly stimulate

tumor cell proliferation (e.g. histamine) [7,66,69,70]. Since mast cells have so many pro- and anti-tumorigenic properties, as well as the ability to change their profiles according to their microenvironment, there are a diverse number of roles that mast cells could potentially play in cancer [7,63,71,72]. Indeed, in prostate cancer, there are conflicting reports as to whether mast cells play a pro- or anti-tumorigenic role in the disease, or if mast cell numbers serve as a positive or negative prognostic factor [8,63].

It has been consistently reported that mast cell numbers are increased in areas of prostate cancer versus benign prostate tissues, and that mast cells are higher in density in lower Gleason grades than in higher Gleason grades [23,24,69,73,74]. However, the literature reporting the prognostic value of mast cells in prostate cancer is far less consistent [8,63], with some studies reporting that high intratumoral mast cell numbers are associated with favorable prognosis [24,74] and some reporting that low intratumoral mast cell numbers are associated with favorable prognosis [75]. Commonly used markers for mast cells include the cell surface receptor c-kit [76] and the most abundant mast cell granule component tryptase [77]. Fleischmann *et al.* stained a 2,300 patient TMA with immunohistochemistry (IHC) for c-kit and reported that higher mast cell densities were significantly associated with longer prostate specific antigen (PSA) recurrence-free survival [24]. Likewise, Johannson *et al.* used IHC for tryptase to identify mast cells in a TMA set of transurethral resection tissues from 394 patients with prostate cancer and also reported that high mast cell density in tumor tissue was associated with longer cancer-specific survival [74]. Interestingly, this group found that in surrounding "peritumoral" benign stroma, higher mast cell densities correlated with shorter cancer-specific survival [74]. Whereas peritumoral mast cell numbers (*e.g.* mast cells directly adjacent

to tumor) have been examined in multiple studies [64,73,74,78], the association between mast cell numbers in benign regions distant to the tumor and outcomes has not been examined.

In contrast to studies reporting an inverse association, Nonomura *et al.* reported that in 104 patient biopsies stained for mast cell tryptase, higher intratumoral mast cell densities were significantly associated with worse PSA-free survival [75]. The differences in findings among studies may be due to differences in study methods such as choice of mast cell marker (*e.g.*, detection of c-kit versus tryptase) and tissue sample type (biopsies versus TMAs). The differences may also relate to variations in mast cell numbers in different regions of the tumor and associated sampling issues, including accounting for benign glands that can be admixed with tumor glands.

Taken together, the association between mast cell numbers or density within the tumor and prostate cancer recurrence remains uncertain. Furthermore, we aimed to examine the association between mast cell numbers or density in benign regions of the prostate distant to the tumor and recurrence. In addition, while most reports have approached the study of mast cells from the perspective of mast cell density in a certain tissue area, we sought to determine whether any observed changes in mast cell numbers were related to either epithelial or stromal area. Thus, in the present study, we evaluated the association between mast cell numbers in both benign and cancerous regions of the prostate and prostate cancer recurrence in men treated by prostatectomy in the "PSA" era (i.e. after PSA testing became widely used and available). We conducted this work among 462 men who recurred (cases) and 462 controls matched to the cases on prognostic factors nested in a cohort of radical

prostatectomy patients at the Johns Hopkins Hospital. We measured the ratio of mast cell number to total tissue area (mast cell density), total epithelial area, or total stromal area in TMAs using an automated digital image analysis tool.

2.3 Methods

Study population, design, and tissue. The design of the nested case-control study of prostate cancer recurrence has been described previously [79]. The sample set included men who underwent radical prostatectomy for clinically localized prostate cancer at The Johns Hopkins Hospital between 1993 and 2001, and had not received hormonal or radiation therapy before radical prostatectomy or adjuvant therapy before recurrence. Cases (n=524) were men who experienced biochemical recurrence as measured by a re-elevation of serum prostate-specific antigen (PSA) ≥0.2 ng/mL, metastasis, or prostate cancer death after surgery by 2004. For each case, a control (n=524) was selected who had not experienced recurrence by the date of the cases' recurrence and who was matched on age, race, pathological stage (T2, T3a, T3b or worse), and Gleason sum (<6, 6, 7, 8+). For these recurrence cases and controls, we previously constructed TMAs containing matched tumor (4 cores, 0.6 mm) and benign tissues (2-4 cores, 0.6 mm). The benign tissue areas used for the TMA construction were generally taken from regions lacking overt inflammatory cell infiltrates when possible. Matched cases and controls were spotted on the same TMA along with various reference (non-prostate) tissues.

Assessment of mast cells. Mast cells were identified by positive IHC staining for tryptase coupled with image analysis.

IHC. We developed an IHC double stain for mast cell tryptase in red and epithelial cell cytokeratin-8 (ck-8) in brown. Slides containing the TMA sections were deparaffinized in xylene and rehydrated through a series of graded ethanol, followed by water and 1% tween in water. The slides were then steamed for 45 minutes in HTTR for antigen retrieval and treated with a dual peroxidase block for 5 minutes. Slides were then incubated at room temperature with 1:40,000 dilution of Abcam mouse anti-tryptase antibody (AA1) and 1:800 dilution of rabbit anti-cytokeratin 8 (ck-8, EP1628Y) for 45 minutes, followed by a cocktail of AP conjugated anti-mouse secondary and HRP-conjugated-anti-rabbit secondary antibody at room temperature for 30 minutes. The slides were then treated with DAB (ck-8 positive cells, brown) and Vector Red (tryptase positive cells, red) for 20 minutes each for visualization. The quality of the IHC was assessed by visualization (*e.g.,* confirming brown staining of epithelium and red staining of stromal mast cells) prior to scanning (20x objective; 0.49 μm per pixel) with an Aperio ScanScope® CS linear-array scanner (Leica Biosystems). After the TMA slides were scanned, the TMA whole slide images were segmented into individual core (JPEG) images using TMALab in eSlideManager (Leica Biosystems). A pathologist examined each core image and an adjacent H&E TMA core image to verify the tissue diagnosis (tumor versus benign) and to document any missing TMA spots that were absent due to technical reasons or because the particular core had been completely cut through.

TMA image analysis. We previously developed PIP (PIP is for Image Processing or PIP, v1.0-rc2, http://www.bitbucket.org/ncuka/pip), software for automated, parallelized image analysis of whole slide images and TMAs [80]. PIP is Java software that uses ImageJ version 1.48e as its processing library [81]. Using the PIP framework, we implemented a custom image analysis algorithm that identified tissue areas, DAB-positive (epithelial) areas and Vector Red-positive cells. Briefly, this algorithm used Hue-Saturation-Balanced (HSB) segmentation to measure DAB-positive areas and a combination of HSB segmentation and color deconvolution for identifying Vector Red-positive mast cells. Core images were concurrently processed in PIP running under Java 8 (version 1.8.0_11). Annotations of each identified region (tissue, DAB) and all mast cells were created for subsequent visual inspection. Algorithm parameters were tuned for use in this high-throughput method for the counting of mast cells within the prostate microenvironment (Figure 2.1A-F). The output of PIP was used to measure the mast cell count, mast cell count per total tissue area (mast cell density), mast cell count per total epithelial area defined as ck-8 positive area (MC/Ep), and mast cell count per stromal area (MC/Stroma) defined as total tissue area minus epithelial area. The image analysis algorithm excluded glandular lumens from tissue and DAB area measurement (see Figure 2.1C). To validate this method, we randomly chose 30 TMA spots (15 benign, 15 tumor) to determine the correlation between the PIP automated count against a "gold standard" manual mast cell count. The automated count and the manual mast cell count for each TMA spot were highly correlated (Pearson correlation coefficient $R^2 = 0.92$, Figure 2.1G). Thus, the PIP software was found to be a valid alternative to manual counting, while allowing for a standardized high-throughput analysis of a large TMA cohort.

Whole slide image analysis. A series of whole tissue sections from 54 men with a range of Gleason sum tumors (6; n=10, 7; n=21, 8; n= 10, 9; n=13) were stained for mast cell tryptase and CK8 and scanned as described above. Each slide was then annotated using Aperio software to circle regions containing tumor. A script was generated to create a 200 micron "mantle" around each tumor to represent the peritumoral region (figure 2.4). The tumor and peritumoral regions were then analyzed for mast cell parameters with the PIP software as described above.

Statistical Analysis. After excluding any missing TMA spots, 478 recurrence cases and 469 controls remained for the tumor tissue spot analysis, and 476 recurrence cases and 474 controls remained for the benign tissue spot analysis. For the primary analysis, we included only complete matched pairs of recurrence cases and controls for tumor tissue spots (462 pairs, 924 spots total) and for benign tissue spots (465 pairs, 930 spots total). We compared means and prevalence of clinical and pathologic characteristics between recurrence cases and controls using the paired t-test and McNemar's test, respectively. Then, for each man, we calculated the mean, minimum, maximum, and standard deviation of mast cell count, mast cell count/total tissue area, mast cell count/total epithelial tissue area, and mast cell count/total stromal tissue area among his up to 4 tumor-containing and among his up to 4 benign tissue TMA spots. We compared these mast cell parameters between the cases and their matched controls using the Wilcoxon-sign-rank test. We divided these mast cell parameters into quartiles based on the distribution in the controls, and then used conditional logistic regression to estimate the odds ratio (OR) of recurrence associated with quartiles of the mast cells parameters taking into account the matching factors. In a sensitivity analysis,

we further adjusted for any differences between the cases and controls in the primary Gleason pattern. The formalin-fixed, paraffin-embedded (FFPE) tissue block age (based on calendar year of surgery) was not associated with mast cell count/total tissue area in cases or controls (data not shown), therefore we did not adjust for block age. In controls, mast cell parameters in cancer and benign tissue were not associated with pre-surgery PSA with the exception of a possible weak association for mast cell count/stromal area (data not shown), therefore we did not adjust for pre-surgery PSA.

In controls, we compared the mast cell parameters between higher-grade (Gleason sum of 4+3 or higher) and lower-grade (Gleason sum 3+4 and lower) disease using the Wilcoxon-rank sum test. Again in controls, we used logistic regression to estimate the OR of higher-grade (versus lower-grade) disease associated with quartiles of the mast cell parameters adjusting for age, race, and pathologic stage. In the series of whole tissue sections, we compared mast cell density between higher-grade and lower-grade disease using the Wilcoxon-rank sum test.

All tests were 2-sided and a p-value <0.05 was considered to be statistically significant. Analyses were performed using SAS release 9.4 (SAS Institute, Cary, NC).

2.4 Results

Recurrence cases and matched controls included in the analysis were similar on the demographic (about 59 years old, majority white), clinical, and pathologic characteristics that were matching factors.

Mast cell density in tumor tissue is lower and in benign tissue is higher in recurrence cases than in controls. For all mast cell parameters assessed, the minimum, rather than the mean, maximum or standard deviation, among a man's TMA spots in tumor and benign tissue was the most robustly different between recurrence cases and controls (Tables 2.1-2.2). Thus, we present results for the minimum throughout. Specifically, the minimum mast cell density among a man's TMA spots was lower in recurrence cases than in controls (Figure 2.1A). In contrast, this minimum in benign tissue was higher in recurrence cases than in controls (Figure 2.2B). These patterns in tumor and in benign tissue were also observed for the minimum of the other mast cell parameters calculated – mast cell count, MC/Ep, MC/Stroma (Tables 2.1-2.2).

In controls, minimum mast cell density did not statistically significantly differ between tumor and benign tissue from the same men, although the mean mast cell density was statistically significantly higher in tumor tissue than in benign tissue (data not shown).

Table 2.1. Mean mast cell distributions* in tumor tissue among prostate cancer recurrence cases and controls, Brady Recurrence Nested Case-Control Study.

	Tumor Tissue						
	Controls			Cases			P-value**
	N	Mean	Standard deviation	N	Mean	Standard deviation	
Mast Cell Count							
Minimum	462	19.12	15.26	462	17.38	15.03	0.003
Maximum	462	41.94	32.4	462	41.07	36.03	0.2
Mean	462	29.82	21.54	462	28.18	22.84	0.02
Standard deviation	440	11.1	11.67	444	11.78	13.25	0.6
Density							
Minimum	462	3.76E-06	3.14E-06	462	3.34E-06	2.87E-06	0.0005
Maximum	462	8.10E-06	6.25E-06	462	7.71E-06	6.28E-06	0.1
Mean	462	5.78E-06	4.26E-06	462	5.34E-06	4.12E-06	0.01
Standard deviation	440	2.12E-06	2.16E-06	444	2.17E-06	2.32E-06	0.9
MC/Ep							
Minimum	462	1.18E-05	1.83E-05	462	9.90E-06	1.20E-05	0.004
Maximum	462	5.54E-05	1.94E-04	462	7.33E-05	5.85E-04	0.08
Mean	462	2.74E-05	6.64E-05	462	3.27E-05	1.94E-04	0.02

Standard deviation	440	2.33E-05	1.12E-04	444	3.30E-05	2.83E-04	0.5
MC/Stroma							
Minimum	462	6.17E-06	4.79E-06	462	5.76E-06	5.27E-06	0.009
Maximum	462	1.38E-05	1.04E-05	462	1.36E-05	1.15E-05	0.2
Mean	462	9.68E-06	6.58E-06	462	9.31E-06	7.46E-06	0.04
Standard deviation	440	3.70E-06	3.83E-06	444	3.86E-06	4.15E-06	0.8

*Most men had two or more TMA spots per tissue type. In the analysis, the minimum, maximum, mean, or standard deviation of each man's TMAs spots was used in the statistical analysis. The sample size for the analysis using the standard deviation is smaller than for the other metrics because some men only had 1 TMA spot for that tissue type. **Wilcoxon Sign Rank Test.

Table 2.2. Mean mast cell distributions* in benign tissue among prostate cancer recurrence cases and controls, Brady Recurrence Nested Case-Control Study.

	Benign Tissue						
	Controls			**Cases**			P-value**
	N	Mean	Standard deviation	N	Mean	Standard deviation	
Mast Cell Count							
Minimum	465	13.95	11.27	465	15.95	10.61	0.0001
Maximum	465	27.67	19.5	465	29.74	19.89	0.07
Mean	465	20.47	13.62	465	22.60	14.10	0.008
Standard	437	8.28	8.31	445	8.57	9.13	

deviation							0.9
Density							
Minimum	465	3.29E-06	2.36E-06	465	3.53E-06	2.21E-06	0.02
Maximum	465	6.31E-06	4.46E-06	465	6.38E-06	4.07E-06	0.6
Mean	465	4.71E-06	2.95E-06	465	4.89E-06	2.84E-06	0.2
Standard deviation	437	1.85E-06	2.18E-06	445	1.74E-06	1.83E-06	0.7
MC/Ep							
Minimum	465	1.53E-05	8.43E-05	465	1.52E-05	2.56E-05	0.002
Maximum	465	1.38E-04	4.25E-04	465	2.13E-04	6.84E-04	0.009
Mean	465	5.84E-05	1.71E-04	465	8.56E-05	1.13E-04	0.002
Standard deviation	437	6.79E-05	2.21E-04	445	1.13E-04	4.01E-04	0.01
MC/Stroma							
Minimum	465	5.16E-6	3.79E-06	465	5.36E-06	3.52E-06	0.2
Maximum	465	1.03E-05	7.14E-06	465	9.85E-06	6.81E-06	0.2
Mean	465	7.57E-06	4.77E-06	465	7.49E-06	4.51E-06	0.7
Standard deviation	437	3.15E-06	3.64E-06	445	2.74E-06	3.15E-06	0.02

*Most men had two or more TMA spots per tissue type. In the analysis, the minimum, maximum, mean, or standard deviation of each man's TMAs spots was used in the statistical analysis. The sample size for the analysis using the standard deviation is smaller than for the other metrics because some men only had 1 TMA spot for that tissue type. ** Wilcoxon Sign Rank Test.

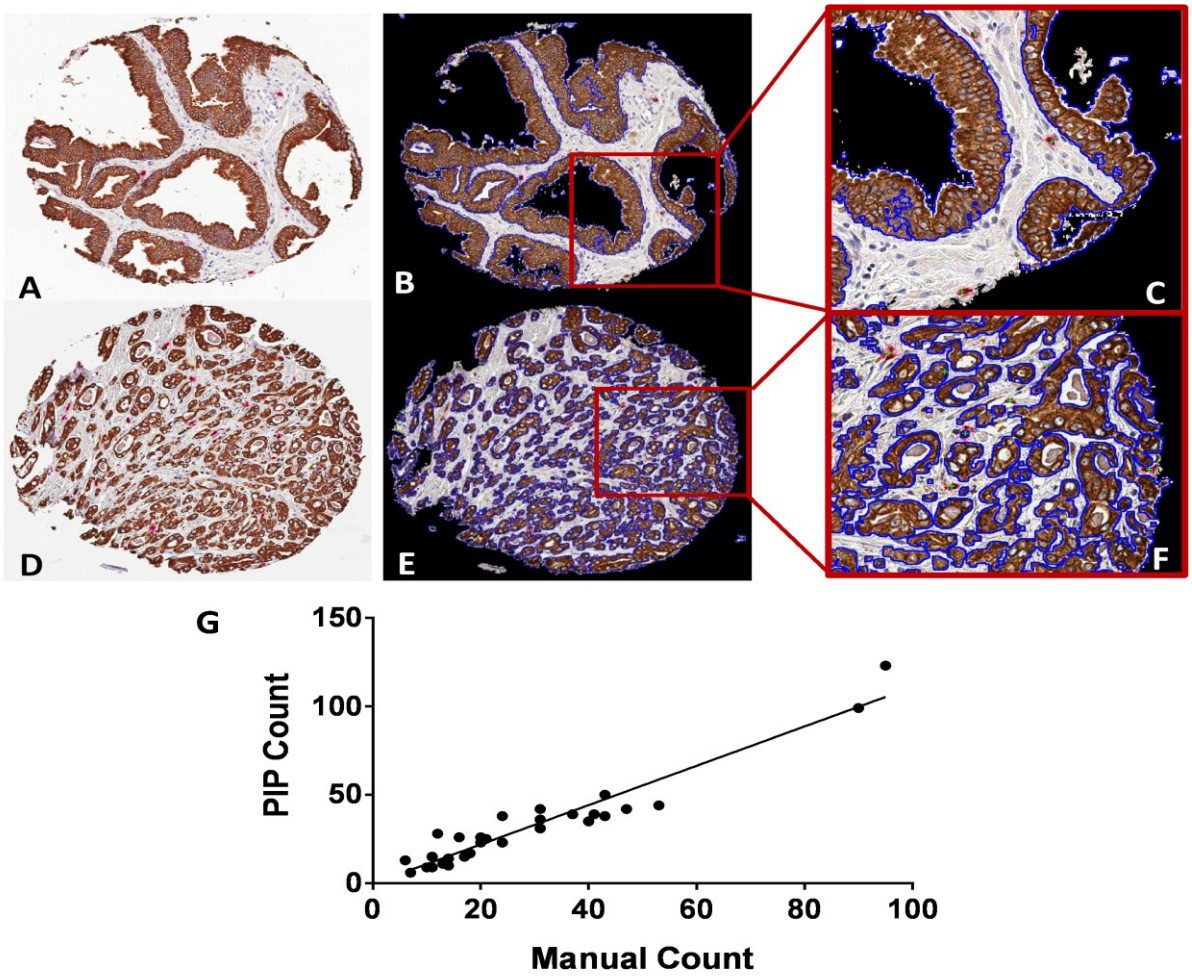


Figure 2.1. PIP software for automated, high-throughput analysis of mast cells in prostate TMAs. TMAs were double immunostained for cytokeratin 8 in DAB (brown) and mast cell tryptase in AP-Vector Red. Stained TMAs were then scanned, segmented into cores and processed using a custom algorithm implemented in PIP. A, D. Examples of IHC double stain of benign and tumor (respectively) prostate tissue spots. B, E. Examples of PIP software processing of prostate tissue TMA spots. Areas without tissue are blacked out, brown ck-8 areas are circled in blue, and red tryptase-positive mast cells are indicated by green markers. C, F. Higher power views of inserts from B and E, respectively. G. Comparison of manual counting of mast cells versus automated counting by the PIP software. R^2=0.92.

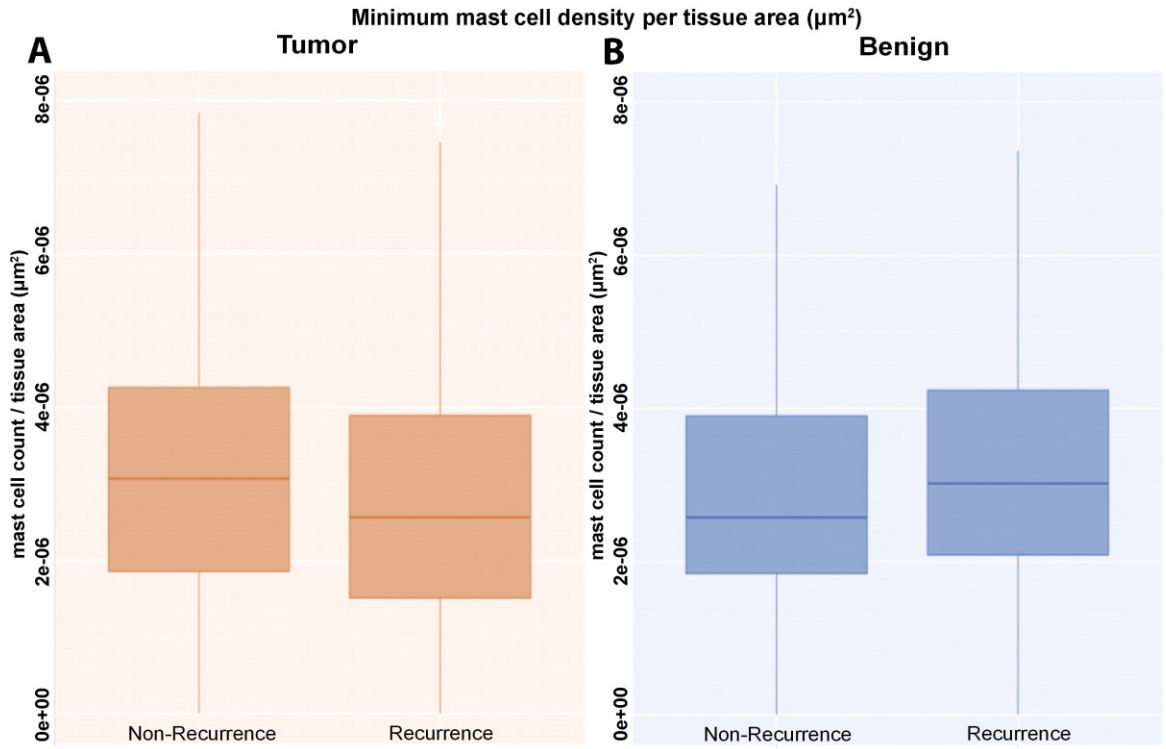


Figure 2.2. Lower minimum mast cell density in tumor and higher minimum mast cell density in benign tissue are associated with prostate cancer recurrence. Graphical representation of mast cell densities in tumor and benign TMA spots from recurrence cases and controls. **A**) Minimum mast cell density in tumor tissue is statistically significantly lower in recurrence cases than controls (p = 0.0005). **B**) Minimum mast cell density in benign tissue is statistically significantly higher in recurrence cases (p = 0.02).

Higher minimum mast cell density in tumor tissue is associated with lower risk of prostate cancer recurrence. Risk of prostate cancer recurrence decreased across quartiles of the minimum of each of the mast cell parameters in tumor (Table 2.3). The strongest inverse association with recurrence was for mast cell density (P-trend=0.004): comparing the highest quartile of mast cell density to the lowest, the OR of recurrence was 0.58 (95% CI 0.40-0.86). Because cases and controls were matched by Gleason sum rather than Gleason pattern, in a sensitivity analysis we additionally adjusted for differences in the primary Gleason pattern. The patterns of association were unchanged (data not shown).

Table 2.3. Association between quartiles of minimum* mast cells parameters in tumor or benign tissue and risk of prostate cancer recurrence, Brady Recurrence Nested Case-Control Study.

	OR of prostate cancer recurrence							
Quartile minimum mast cell parameter**	**Tumor tissue**				**Benign tissue**			
Mast Cell Count	**No. of recurrence cases/controls**	**OR**	**95% CI**	**P-value**	**No. of recurrence cases/controls**	**OR**	**95% CI**	**P-value**
1 (lowest)	154/126	1	Reference		96/121	1	Reference	
2	134/118	0.87	(0.61-1.23)		89/120	0.96	(0.66-1.40)	
3	77/109	0.53	(0.36-0.80)		115/114	1.27	(0.88-1.83)	
4 (highest)	97/109	0.66	(0.44-0.98)		165/110	2.07	(1.41-3.05)	
		P-trend		0.02				<0.001
Density								
1 (lowest)	150/116	1	Reference		98/114	1	Reference	
2	126/115	0.81	(0.57-1.16)		84/117	0.81	(0.55-1.19)	
3	94/117	0.6	(0.41-0.87)		140/120	1.36	(0.95-1.93)	
4 (highest)	92/114	0.58	(0.40-0.86)		143/114	1.48	(1.02-2.14)	
		P-trend		0.004				0.007
MC/Ep								
1 (lowest)	152/116	1	Reference		88/117	1	Reference	

2	104/114	0.67	(0.46-0.97)	109/115	1.27	(0.87-1.85)
3	110/117	0.68	(0.47-0.99)	116/114	1.41	(0.96-2.08)
4 (highest)	96/115	0.6	(0.41-0.88)	152/119	1.79	(1.22-2.64)
		P-trend	0.03			0.004
MC/Stroma						
1 (lowest)	150/116	1	Reference	104/117	1	Reference
2	126/115	0.81	(0.57-1.16)	97/113	0.96	(0.66-1.41)
3	94/117	0.6	(0.41-0.87)	126/117	1.2	(0.84-1.71)
4 (highest)	92/114	0.58	(0.40-0.86)	138/118	1.32	(0.92-1.91)
		P-trend	0.01			0.07

*Minimum value for a given mast cell parameter among each man's TMA spots. **Quartile cut points based on control group, matched on age, race, pathologic stage, and pathologic Gleason sum, P-trend calculated using median of quartiles.

Higher minimum mast cell density in benign tissue is associated with a higher risk of prostate cancer recurrence. In contrast to tumor, risk of prostate cancer recurrence increased across quartiles of the minimum of each of the mast cell parameters in benign tissue (Table 2.3). The strongest positive association with recurrence was for mast cell count (P-trend<0.001): comparing the highest quartile of mast cell density to the lowest, the OR of recurrence was 2.07 (95% CI 1.41-3.05).

Risk of recurrence is highest for the combination of lower minimum mast cell density in tumor tissue and higher minimum mast cell density in benign tissue compared with all other combinations. Given the opposite direction of the associations with recurrence for high mast cell parameters in tumor and benign tissue, we next evaluated the association of mast cell parameters in the combination of tumor and benign tissue with recurrence. Low density in tumor tissue and high density in benign tissue was associated with a higher risk of recurrence compared with all other combinations (OR=1.87, 95% CI 1.36-2.58). However, no statistical interaction between mast cell density in tumor and benign tissue was present (P=0.2).

Mast cell parameters in tumor, but not in benign tissue, are inversely associated with Gleason sum in controls. Given prior studies, we also investigated whether the mast cell parameters differed by Gleason sum (data not shown) and are associated with higher-grade disease in the controls. The OR of higher-grade disease increased across quartiles of the minimum mast cell count (P-trend=0.02), mast cell density (P-trend=0.01, Figure 2.3A), mast cell count to epithelial area (P-trend=0.02), and mast cell count to stromal area (P-trend=0.02) in tumor (Table 2.4). The OR of higher-grade disease did not change across minimum mast cell parameters in benign tissue (Figure 2.3B, Table 2.4).

Table 2.4. Association of quartiles of minimum* number of mast cells in tumor and in benign prostate tissue with Gleason sum, controls, Brady Recurrence Nested Case-Control Study.

	OR of higher-grade prostate cancer***					
Quartile minimum mast cell parameter**	**Tumor tissue**			**Benign tissue**		
Mast Cell Count	**No. higher-/lower-grade prostate cancer**	**OR**	**95% CI**	**No. higher-/lower-grade prostate cancer**	**OR**	**95% CI**
1 (lowest)	154/126	1	Reference	96/121	1	Reference
2	134/118	0.56	(0.29-1.05)	89/120	0.62	(0.32-1.22)
3	77/109	0.51	(0.25-1.01	115/114	1.11	(0.58-2.12)
4 (highest)	97/109	0.42	(0.21-0.86)	165/110	0.48	(0.23-0.99)
	P-trend		0.02			0.1
Density						
1 (lowest)	150/116	1	Reference	98/114	1	Reference
2	126/115	0.31	(0.15-0.64)	84/117	0.98	(0.49-1.94)
3	94/117	0.39	(0.20-0.75)	140/120	1.14	(0.59-2.23)
4 (highest)	92/114	0.4	(0.20-0.78)	143/114	0.62	(0.30-1.30)
	P-trend		0.01			0.2
MC/Ep						

1 (lowest)	152/116	1	Reference	88/117	1	Reference
2	104/114	0.76	(0.52-1.11)	109/115	1.05	(0.52-2.10)
3	110/117	0.81	(0.55-1.20)	116/114	1.1	(0.55-2.21)
4 (highest)	96/115	0.64	(0.43-0.95)	152/119	0.8	(0.39-1.65)
		P-trend	0.02			0.7
MC/Stroma						
1 (lowest)	150/116	1	Reference	104/117	1	Reference
2	126/115	0.33	(0.16-0.66)	97/113	0.55	(0.27-1.09)
3	94/117	0.57	(0.30-1.06)	126/117	0.7	(0.36-1.36)
4 (highest)	92/114	0.36	(0.18-0.74)	138/118	0.55	(0.92-1.97)
		P-trend	0.02			0.5

*Minimum value for a given mast cell parameter among each man's TMA spots. **Quartile cut points based on the distribution in the controls. P-trend calculated using the median of quartiles in the controls. Model adjusted for age, race, and pathologic stage. ***Higher-grade (≥ Gleason 4+3) versus lower-grade (≤ Gleason 3+4).

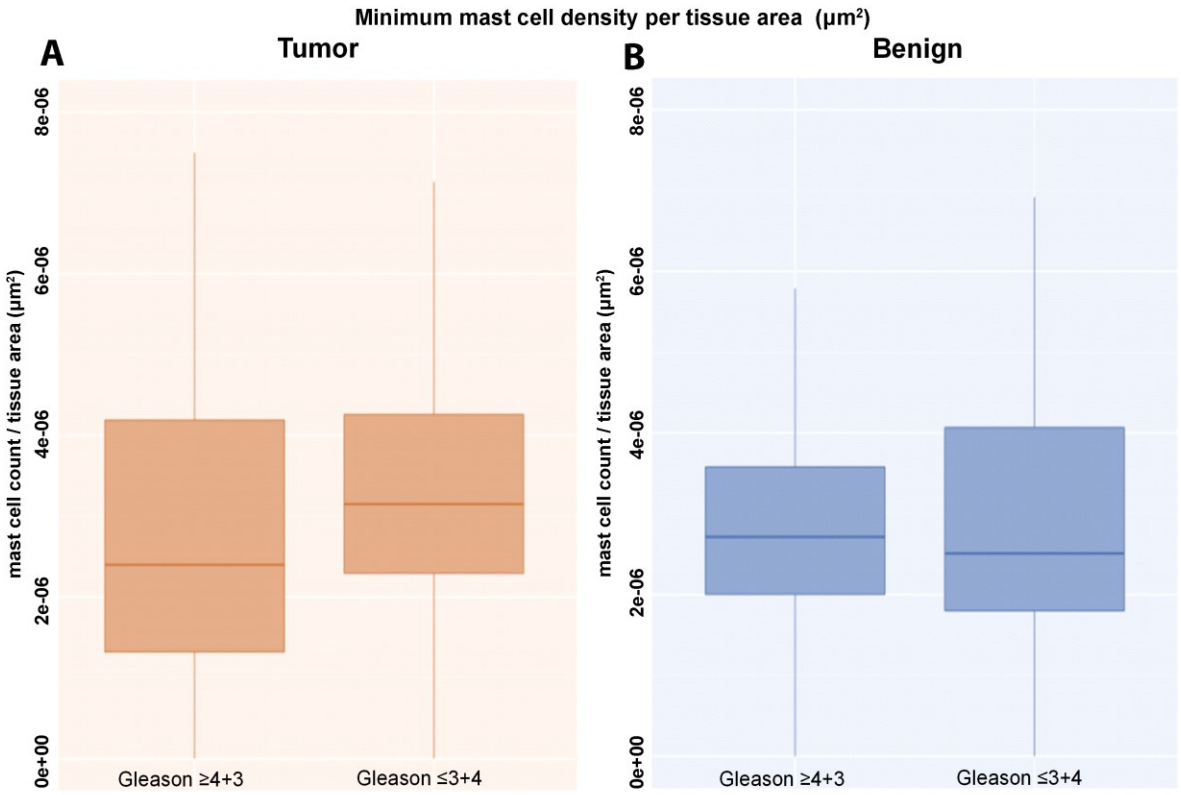


Figure 2.3. Minimum mast cell density is higher in lower Gleason sum tumors. This analysis was done in the controls. Graphical representation of minimum mast cell density in high (≥4+3=7) vs low (≤3+4=7) Gleason sum tumors. **A**) Minimum mast cell density in tumor tissue is statistically significantly higher in lower Gleason sum tumors (p=0.004). **B**) Minimum mast cell density in benign tissue is not different between high and low Gleason sum tumors (p=0.6).

In the analysis of mast cell density in tumor versus peritumoral regions in the 54 whole tissue sections (Figure 2.4), we also observed significantly lower mast cell density in higher grade tumors (Gleason >7, P=0.006), and this pattern was also observed for the peritumoral regions analyzed (P=0.007, Figure 2.4). Although not statistically significant (P=0.3), lower mast cell densities were observed in higher grade tumors when separated by Gleason 3+4 or lower versus Gleason 4+3 or higher as we found in the recurrence TMA.

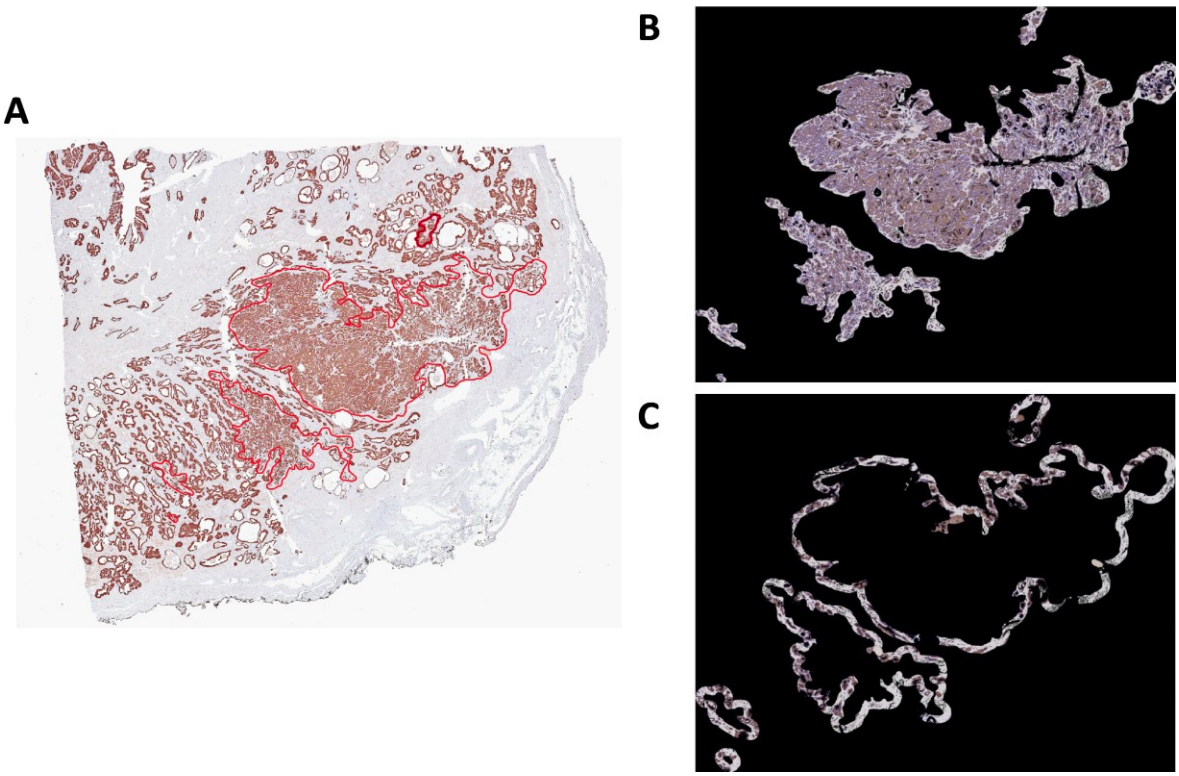


Figure 2.4. Whole slide image analysis. A) Section stained for CK8/tryptase where tumor is circled in red using Aperio annotation software. Image analysis of **B**) isolated tumor area and **C**) peritumoral region.

2.5 Discussion and conclusions

In this study of surgically treated men with clinically localized prostate cancer, we found that higher numbers of mast cells in tumor was associated with a lower risk of prostate cancer recurrence taking into account stage and grade at diagnosis. The most robust findings were observed when using the minimum mast cell count among each man's TMA spots assessed. Our results suggest that intratumoral mast cells may be protective against prostate cancer recurrence and could potentially serve as a prognostic biomarker after prostatectomy.

The inverse association with recurrence was also observed when standardizing mast cell count for area of total, epithelial, and stromal tissue. We hypothesized that mast cells

may maintain a certain ratio to epithelial cells, and that therefore mast cell numbers may be higher in cancer due to the higher epithelial cell to stromal cell ratio in cancer. However, the association for mast cell density (mast cell count to total tissue area) was more strongly inverse than for mast cell count to epithelial area. Thus, mast cell density may be the most useful measure of mast cell abundance in the context of prostate cancer recurrence.

There are a number explanations as to why higher densities of intratumoral mast cells could be inversely associated with recurrence. Mast cells express MHC II to present antigens as well as the costimulatory molecule CD28, and are known to recruit neutrophils, eosinophils, macrophages, and B and T cells capable of mounting an anti-tumor immune response [65-67]. Mast cells are also capable of producing molecules such as IL-4 and TNF-α that can have a negative impact on tumor cell growth and survival directly [7,82]. In addition, it is possible that prostate cancer cells that are more likely to lead to recurrence undergo a molecular switch that prevents the recruitment of mast cells in response to the cancer, resulting in mast cells being a proxy marker for poor prognosis. The biological role of intratumoral mast cells and their possible use as a prognostic factor is worth further investigation.

We also found that a higher number of mast cells in benign tissue was associated with a higher risk of prostate cancer recurrence. There are several possible explanations as to why mast cells in benign regions of a prostate that contains cancer could be associated with a greater likelihood of recurrence. Mast cells are highly pro-angiogenic and may serve to promote a pro-tumorigenic microenvironment and also promote metastasis [66]. Mast cells are also theorized to promote tumor invasion via production of MMP9 and other collagenases, as well as proteases and other effector molecules capable of changing the tissue architecture

[8,65,66,68]. Mast cells are also capable of synthesizing and secreting a number of factors that may promote tumor proliferation, including histamine, NGF, SCF, and IL-8 [63]. Mast cells are also believed to recruit regulatory T cells (T_{regs}) and promote tumor immune tolerance [66,70,83]. Finally, higher mast cell numbers in benign regions of the prostate could serve as a proxy for a higher degree of chronic inflammation. Men with a higher degree of chronic inflammation in the benign regions the prostate may in turn be more likely to recur. Indeed, chronic inflammation in benign prostate tissues has been found to be associated with higher grade prostate cancer [84]. One limitation of our study is that the benign areas used for the TMA construction were taken from region lacking overt inflammatory cell infiltrates when possible. To determine the generalizability of our findings, future studies should examine mast cell numbers in benign regions in the prostate sampled randomly.

This study also sought to confirm prior studies showing that mast cell density is inversely associated with Gleason sum in cancer tissues, and we additionally assessed the association between mast cell density in benign tissues and Gleason sum. Our findings in the controls were consistent with prior studies in that cancer tissue mast cell densities as well as mast cell count, mast cell count/epithelial area, and mast cell count/stromal area were significantly higher in men with lower Gleason sum (≤3+4) than in higher Gleason sum (≥4+3) cancers. A similar finding was observed in whole tissue sections from a range of Gleason sum tumors analyzed for both tumor and peritumoral regions, but only when higher-grade tumors were defined as Gleason sum >7. This result supports that TMAs may provide similar mast cell parameters information as whole tissue sections. We suspect the 3+4 versus 4+3 analysis was underpowered in this small cohort.

In contrast, mast cell count in benign tissue was not associated with Gleason sum. These inconsistent associations between tumor and benign tissue suggest that mast cell alterations in the context of Gleason sum are specific to the tumor microenvironment, and not inherent to the prostate as a whole. Whether increased or decreased mast cell density in tumor tissue is related to prostate cancer outcome is still somewhat controversial in that there are mixed results reported so far [24,74,75]. Our finding that a lower mast cell count in tumor tissue is associated with worse outcome independent of standard clinical and pathological variables (e.g. pathologic Gleason score and pathologic stage at prostatectomy) is consistent with those reported by Fleischmann *et al*., [24] and Johannson *et al*. [74] and together suggest that there may be an anti-tumor biological role for mast cells in prostate cancer tissue. We do not know why the minimum mast cell parameter of each man's TMA spots was the most robustly different measure between recurrence cases and controls. We do know that mast cell numbers within different regions of the tumor could vary depending on a number of factors including the presence of other inflammatory cells and/or immune stimuli and local cytokine production. Thus, if intratumoral mast cells indeed do protect against recurrence, we speculate that the region with the lowest number (rather than the average number, the maximum number, or the variability in number across regions of the tumor) may provide the most information about risk of recurrence.

Key strengths of our study include the large, well-characterized patient cohort coupled with the use of newly developed automated image analysis for unbiased detection and quantification of mast cells, tissue area, epithelial area, and stromal area on thousands of TMA spots. To support the clinical utility of our findings, mast cells in tumor and benign

areas sampled without respect to overt inflammation should be further investigated in additional prostate cancer patient populations.

In summary, using a novel image analysis tool we report that a low number of intratumoral mast cells is associated with of a higher risk of prostate cancer recurrence. Furthermore, we observed that a high number of mast cells in benign tissue appears to be associated with a higher risk of recurrence. These findings suggest further investigations both to better understand mast cell biology in prostate cancer and to determine clinical utility.

III. MAST CELLS IN PROSTATE CANCER RACE DISPARITIES AND METASTASIS

3.1 Abstract

Prostate cancer presents with a significant racial disparity, with African American (AA) men being twice as likely to be diagnosed with prostate cancer, and twice as likely to die from their disease. AA men are also typically diagnosed with prostate cancer at an earlier age and their tumors tend to be more aggressive (e.g. higher Gleason grade and stage) at diagnosis. Finally, biopsies from AA men have a higher prevalence and degree of inflammation. Given our previous results that low intratumoral mast cell densities are associated with greater rates of PSA progression (Chapter II), we next explored whether prostate cancer in AA men contains lower densities of intratumoral mast cells compared to Caucasian American (CA) men, and whether intratumoral mast cell densities in AA men are similarly associated with an increased risk of biochemical recurrence. Our previous study contained primarily tumors from CA men. We used multiple cohorts using TMAs constructed with tumor and benign

prostate tissues from AA and CA men matched on cancer grade and cancer stage. Our results showed that intratumoral mast cell densities were significantly lower in tumors of AA men in comparison to CA men specifically in low Gleason score cancers (Gleason ≤ 3+4=7). The pattern observed for low numbers of intratumoral mast cells and biochemical recurrence in the PSA Progression TMA set was present in all strata in both CA and AA men in the present study. This association remained when development of metastasis was the clinical endpoint. Thus, these results indicated that the same relationship between low numbers of intratumoral mast cells and worse prostate cancer outcomes was present for AA men, and additionally identified a potentially important relationship between low numbers of intratumoral mast cells and the development of metastases.

3.2 Introduction

Prostate cancer afflicts AA men to a greater degree than CA men, with AA men having significantly higher serum PSA levels, higher likelihood of positive surgical margins, 60% greater incidence of prostate cancer, and 2-3 times higher mortality rates [85-89]. The cause(s) of this racial disparity are unclear, and many mechanisms have been suggested including societal factors such as access to healthcare, Social Economic Status (SES), and lack of medical intervention due to distrust of the medical community [85,90-92]. While all these factors may contribute to the overall problem, there is evidence to suggest that these factors do not fully explain the racial disparity [93]. Intriguingly, focused studies in AA cohorts have revealed markedly different frequencies of somatic alteration in several genes. For example, *ERG* rearrangements have been observed in approximately half of all prostate tumors

occurring in CA men[94], but these rearrangements are only seen in 25-30% of tumors in AA men [95,96]. These data suggest that there are also biological differences in tumors from AA men. As such, many studies have further explored a biological explanation for racial disparities in prostate cancer. For example, Powell *et al* evaluated autopsy specimens of African American (AA) and European American (EA) men who died of causes unrelated to prostate cancer, as well as radical prostatectomy specimens of men diagnosed with cancer [87]. Their findings showed that AA men had similar rates of preclinical prostate cancer and similar preclinical characteristics as EA men [87]. However, radical prostatectomy specimens show a significantly higher tumor volume and Gleason Score in AA than EA men [87]. In addition, the authors found that AA men were significantly more likely to have advanced or metastatic disease than EA men, with a ratio of 4:1 [87]. These findings indicate that prostate cancer in AA men has a higher rate of growth and may be more aggressive than in CA men. Similarly, an additional study found AA men to have a 61% likelihood of significant prostate cancer as compared to only 27% in CA men, as well as a significantly higher likelihood of a Gleason score of 7 or higher and a tumor volume greater than $0.5cm^3$ [89]. These studies did not, however, implicate a particular mechanism or genetic susceptibility explaining this higher growth rate and metastatic potential.

Evidence is mounting for a role for inflammation in prostate cancer, with inflammation being frequently observed upon histological examination of prostate biopsies, radical prostatectomy specimens, and autopsy prostate specimens [97]. In addition, inflammation is proposed to drive the development of a putative precursor lesion termed proliferative inflammatory atrophy (PIA), which may have the potential to develop into prostatic intraepithelial neoplasia (PIN) and then to adenocarcinoma [98-100]. Finally,

inflammatory cells and mechanisms of immune modulation and tolerance have been suggested to play key roles in castration resistance [101-103]. As such, there is significant possibility that higher rates of inflammation would be associated with higher rates of prostate cancer, as well as greater potential for advanced or metastatic disease. Interestingly, studies seeking to elucidate a mechanism to explain the racial disparity of prostate cancer are increasingly highlighting a differential rate of inflammation in the prostates of AA compared to CA men.

AA men have been shown to have an increased prevalence of inflammation in prostate biopsies free of prostate cancer compared to CA men [86]. In addition, microarray studies have identified differential gene expression profiles in prostate tumors from AA men compared to CA men that demonstrate a pattern of high representation of genes associated with inflammatory pathways, including IL-10, PTPN22, components of HLA complex, TAP 1 and 2, TCR, IL4, IL13, CXCR4, CCL5/CCR5, and MMP9 [88,93,104,105]. Of particular interest, many of the genes identified as being higher expressed in tumors of AA men, namely IL4, IL13, CXCR4, CCL5/CCR5, and MMP9, are associated with chemotaxis and function of mast cells [106-109].

Mast cells are immune cells resident in most tissues of the body, including the prostate, and are known to secrete effector molecules such as IL4, IL13, histamine, VEGF, and MMP9 [109,110]. In addition, CXCR4 and CCR5 are known to mediate mast cell progenitor chemotaxis [109]. As was discussed in Chapters I and II, mast cells potentially contribute to angiogenesis, invasion, immune modulation, and tumor cell proliferation, and are highly pro-inflammatory [70,110-116]. As reported in Chapter II, we analyzed mast cell density in relation to biochemical recurrence in a cohort of radical prostatectomy patients using TMAs that

sampled both tumor and matched benign tissue [120]. Our findings indicated that higher intratumoral mast cell density was associated with a lower risk of recurrence, while higher benign tissue mast cell density was associated with a higher risk of recurrence[120].

Due to the evidence suggesting a differential presence of prostatic inflammation in AA and CA men, as well as our previous data suggesting a relationship between mast cells and recurrence in men with prostate cancer, we investigated the potential role for mast cells in the race disparity of prostate cancer. We used a similar approach as outlined in Chapter II, thereby analyzing the maximum, median, and minimum mast cell density counts for tissues from AA and CA men. We utilized three different cohorts and TMAs representing tumor and benign prostate tissues from both AA and CA men. For one cohort in which outcomes data was available, we assessed mast cell density and correlated with risk of recurrence and development of metastases. In addition, distant metastases from rapid autopsy tissues from three prostate cancer patients were stained for tryptase to evaluate intratumoral mast cell density in metastatic lesions.

3.3 Methods

IHC Staining. Slides containing the TMA sections were deparaffinized in xylene and rehydrated through a series of graded ethanol, followed by water and 1% tween in water. The slides were then steamed for 45 minutes in HTTR for antigen retrieval and treated with a dual peroxidase block for 5 minutes. Slides were then incubated at room temperature with 1:32,000 dilution of Abcam mouse anti-tryptase antibody (AA1) and/or 1:800 dilution of rabbit anti-cytokeratin 8 (ck-8, EP1628Y) for 45 minutes, followed by a cocktail of AP

conjugated anti-mouse secondary and HRP-conjugated-anti-rabbit secondary antibody at room temperature for 30 minutes. The slides were then treated with DAB (ck-8 positive cells, brown) and/or Vector Red (tryptase positive cells, red) for 20 minutes each for visualization.

TMA Sets:

The following TMA sets were used in the present study:

TMA Set	**Description**
150 Case Low Grade Race Disparity	Radical prostatectomy cohort of 75 AA men matched to 75 CA men on age +/- 3 years, grade and stage. Contains primarily Gleason 6 and 7 tumors. Analyzed both tumor and matched benign tissues.
338 Case Race Disparity	Radical prostatectomy cohort of 169 AA men matched to 169 CA men selected via stratified random sampling among Gleason score groupings (3+3, 3+4, 4+3, 8, 9-10). Analyzed both tumor and matched benign tissues. Outcomes data available.
120 Case High Grade Race Disparity	Radical prostatectomy cohort of 60 AA men matched to 60 CA men on age +/- 3 years, grade and stage. Enriched for cases with Gleason ≥8. Analyzed both tumor and matched benign tissues.

Collection and preparation of rapid autopsy tissues. Metastatic tissues were obtained at autopsy, fixed in 10% buffered formalin for 48 hours, and then paraffin embedded and sectioned. Bone metastatic samples were decalcified before formalin fixation and embedding.

Scanning and analysis with PIP software. See PIP software development in Chapter II. The PIP software was used to measure the number of mast cells per TMA spot (mast cell count or MC) and mast cell density (MC/tissue area).

Statistical Analysis. A similar approach was taken as outlined in Chapter II. Statistical analysis was performed using SAS release 9.4 (SAS Institute, Cary, NC) with the analyses indicated in the table legends.

3.4 Results

We first aimed to determine whether there is a significant difference in mast cell number or density in tumor or matched benign prostate tissues from AA and CA men. This was accomplished through immunostaining and analysis of three TMA sets: 150 Case Low Grade Race Disparity, 120 Case High Grade Race Disparity, and a 338 Case Race Disparity (see Methods). There were no significant differences in mast cell density or number in tumor or benign tissues in AA men compared to CA men in the 120 Case High Grade Race Disparity TMA set (Table 3.1). However, the 150 Case Low Grade Race Disparity TMA set showed significantly lower densities of mast cells in tumor tissues in AA men compared to CA men, but no differences were observed in mast cell density in benign tissues between AA and CA men (Table 3.2). A similar relationship was observed in the 338 Case Race Disparity TMA set when stratified by Gleason score; tumors with low Gleason scores (Gleason ≤ 3+4=7) showed significantly lower mast cell densities in the tumors of AA versus CA men, while high Gleason score tumors showed no significant difference (Table 3.3).

Table 3.1. Comparison of mast cell density and count in AA versus CA men in the 120 Case High Grade Race Disparity TMA set.

		Mast Cell Density						Mast Cell Count				
Tumor Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD

CA	60	1.8	4.4	3.2	2.9	1.1		10.0	26.5	19.5	17.8	6.9
AA	57	2.1	4.2	3.1	3.1	1.0		11.0	24.0	18.3	17.0	5.5
p		0.6	0.6	0.9	0.9	0.3		0.8	0.4	0.7	0.7	0.1
OR (95% CI)		1.1 (0.5-2.6)	1.1 (0.5-2.6)	1.3 (0.5-2.9)	1.3 (0.6-3.1)	0.9 (0.4-2.2)		1.2 (0.5-2.8)	1.0 (0.4-2.3)	1.2 (0.5-2.9)	1.3 (0.5-3.1)	0.8 (0.3-2.1)
Normal Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	60	1.7	4.7	3.1	3.1	1.3		8.5	25.5	16.9	16.0	6.8
AA	60	1.8	4.4	3.1	2.9	1.0		9.0	24.0	16.1	14.5	6.2
p		0.8	0.7	0.8	0.5	0.3		0.5	0.9	0.9	0.5	0.7
OR (95% CI)		1.6 (0.6-3.9)	1.2 (0.5-2.9)	1.7 (0.7-4.0)	1.6 (0.7-3.7)	1.2 (0.5-3.1)		1.5 (0.6-3.6)	1.3 (0.5-3.1)	1.6 (0.7-3.7)	1.1 (0.5-2.6)	1.2 (0.5-2.9)

p: From Wilcoxon signed-rank test
OR (odds ratio, AA vs. CA): For minimum, odds of falling below first quartile of the respective mast cell measure in CA men. For the other measures, odds of falling above third quartile of the respective mast cell measure in CA men. Logistic regression model is adjusted for age, stage, grade and surgical margin status.

Table 3.2. Comparison of mast cell density and count in AA versus CA men in the 150 Case Low Grade Race Disparity TMA set.

		Mast cell density						Mast cell count				
Tumor Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	75	2.5	4.6	3.4	3.3	1.1		13.0	26.0	19.3	19.0	6.5
AA	76	1.9	4.1	3.1	2.9	0.9		11.0	24.0	18.0	16.0	5.5
p		0.1	0.03	0.02	0.02	0.1		0.1	0.1	0.1	0.03	0.4
OR (95% CI)		1.8 (0.9-3.8)	0.4 (0.2-0.9)	0.4 (0.1-0.9)	0.4 (0.2-0.98)	0.7 (0.3-1.7)		1.1 (0.6-2.3)	0.6 (0.2-1.3)	0.5 (0.2-1.2)	0.5 (0.2-1.1)	0.7 (0.3-1.6)
Normal Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	75	1.8	4.0	3.0	3.1	1.0		14.0	21.0	15.5	15.0	5.5

AA	77	1.4	4.1	2.8	2.7	0.9		7.0	20.0	13.8	13.0	4.9
p		0.1	0.2	0.1	0.03	0.2		0.04	0.1	0.04	0.02	0.1
OR (95% CI)		2.0 (0.96-4.0)	0.7 (0.3-1.6)	0.3 (0.1-0.7)	0.4 (0.2-1.1)	0.8 (0.4-1.7)		1.8 (0.9-3.5)	0.5 (0.2-1.3)	0.4 (0.1-0.99)	0.4 (0.2-1.1)	0.6 (0.3-1.4)
p: From Wilcoxon signed-rank test OR (odds ratio, AA vs. CA): For minimum, odds of falling below first quartile of the respective mast cell measure in CA men. For the other measures, odds of falling above third quartile of the respective mast cell measure in CA men. Logistic regression model is adjusted for age, stage, grade and surgical margin status. Statistically significant values are highlighted in red.												

Table 3.3. Comparison of mast cell density and count in AA versus CA men in the 338 Case Race Disparity TMA set.

Gleason=3+4 or below		Mast cell density						Mast cell count				
Tumor Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	72	3.0	5.2	4.0	4.1	1.3		10.0	29.0	22.0	23.3	7.9
AA	72	2.5	5.5	3.9	3.9	1.4		15.0	31.0	23.8	23.5	8.6
p		0.05	0.8	0.5	0.4	0.2		0.3	0.7	0.8	0.7	0.5
OR (95% CI)		3.6 (1.4-9.4)	1.0 (0.4-2.4)	0.6 (0.2-1.4)	0.7 (0.3-1.7)	2.6 (1.1-6.3)		1.8 (0.8-4.4)	1.0 (0.4-2.4)	0.7 (0.3-1.6)	0.6 (0.3-1.5)	1.4 (0.6-3.1)
Normal Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	73	2.7	7.1	4.6	4.3	1.6		12.0	33.0	21.8	21.5	7.8
AA	73	2.3	4.7	3.4	3.5	1.3		10.0	22.0	17.5	17.0	6.4
p		0.04	0.01	0.01	0.01	0.1		0.2	0.01	0.02	0.02	0.05
OR (95% CI)		1.9 (0.9-4.2)	0.6 (0.3-1.5)	0.3 (0.1-0.8)	0.4 (0.2-1.1)	0.8 (0.4-1.9)		1.2 (0.5-2.5)	0.5 (0.2-1.3)	0.3 (0.1-0.8)	0.4 (0.1-0.98)	0.7 (0.3-1.6)
Gleason=4+3 or above		Mast cell density						Mast cell count				
Tumor Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD

CA	74	2.0	5.4	3.6	3.7	1.4		13.0	30.5	20.8	21.0	8.1
AA	61	2.1	5.0	3.1	3.2	1.5		11.0	31.0	18.0	18.0	9.4
p		0.9	0.7	0.8	0.8	0.9		0.8	0.9	0.7	0.7	0.5
OR (95% CI)		0.9 (0.4-1.9)	0.8 (0.3-1.8)	1.2 (0.5-2.9)	1.0 (0.4-2.4)	1.0 (0.4-2.2)		0.9 (0.4-1.9)	0.9 (0.4-2.2)	1.0 (0.4-2.4)	1.1 (0.5-2.6)	1.3 (0.6-3.1)
Normal Tissue	N	Min ($x10^{-6}/\mu m^2$)	Max ($x10^{-6}/\mu m^2$)	Mean ($x10^{-6}/\mu m^2$)	Med ($x10^{-6}/\mu m^2$)	STD ($x10^{-6}/\mu m^2$)		Minimum	Maximum	Mean	Median	STD
CA	77	2.4	5.1	3.7	3.9	1.2		11.0	26.0	19.3	19.0	6.7
AA	62	2.4	6.0	4.3	4.2	1.5		10.0	31.0	20.8	19.8	8.6
p		0.9	0.4	0.5	0.5	0.1		0.8	0.5	0.7	0.6	0.1
OR (95% CI)		0.8 (0.4-1.8)	1.7 (0.7-3.9)	1.3 (0.5-3.0)	1.2 (0.5-2.8)	2.3 (0.99-5.1)		1.0 (0.4-2.3)	1.7 (0.8-3.9)	1.4 (0.6-3.5)	1.4 (0.6-3.3)	1.9 (0.8-4.2)

p: From Wilcoxon signed-rank test
OR (odds ratio, AA vs. CA): For minimum, odds of falling below first quartile of the respective mast cell measure in CA men. For the other measures, odds of falling above third quartile of the respective mast cell measure in CA men. Logistic regression model is adjusted for age, stage, grade and surgical margin status. Statistically significant values are highlighted in red.

Next, we determined whether mast cell numbers and/or density in prostate tumor and benign tissues from AA men had a similar relationship with risk of prostate cancer recurrence (PSA progression) as we previously observed (Chapter II), where higher numbers of intratumoral mast cells were associated with lower risk of recurrence. Since our previous cohort was comprised predominantly of tissues from CA men, we tested our hypothesis using the 338 Case Race Disparity TMA set which included outcome data, such as biochemical recurrence and development of metastases after radical prostatectomy. The pattern observed for low numbers of intratumoral mast cells and biochemical recurrence in the PSA Progression TMA set (Chapter II) was present in all strata in both CA and AA men in the

present study, although not statistically significant (Table 3.4). Interestingly, these associations remained, and appeared even stronger, when development of metastasis was the outcome (Table 3.5).

Table 3.4. Adjusted Hazard Ratio* (HR) of prostate cancer biochemical recurrence by race and mast cell measurements, 338 Case Race Disparity TMA.

All	Tumor Tissue				Benign Tissue			
Minimum Mast Cell Density	Case/Control	HR	95% CI	p	Case/Control	HR	95% CI	p
1(Lowest)	36/29	1			22/36	1		
2	18/37	0.91	0.48-1.71		24/38	1.13	0.59-2.15	
3	17/42	0.87	0.45-1.70		29/28	1.13	0.61-2.10	
4(Highest)	17/28	0.76	0.40-1.44		12/34	0.74	0.35-1.58	
	P trend			0.4				0.5
1(Lowest)	36/29	1			22/36	1		
2,3 and 4	52/107	0.84	0.51-1.38		65/100	1.02	0.60-1.76	
Minimum Mast Cell Count								
1(Lowest)	34/28	1			24/34	1		
2	19/41	0.74	0.40-1.35		22/42	1.05	0.56-1.99	
3	16/42	0.78	0.39-1.53		30/30	1.06	0.59-1.91	
4(Highest)	19/25	0.87	0.48-1.59		11/30	0.68	0.32-1.45	
	P trend			0.6				0.5
1(Lowest)	34/28	1			24/34	1		
2,3 and 4	54/108	0.79	0.49-1.27		63/102	0.96	0.57-1.61	

CA

Minimum Mast Cell Density								
1(Lowest)	21/10	1			12/18	1		
2	7/24	0.74	0.28-1.92		12/20	1.28	0.54-3.01	
3	8/25	0.56	0.23-1.35		15/17	1.13	0.48-2.68	
4(Highest)	12/19	0.72	0.33-1.59		9/22	1.15	0.46-2.87	
	P trend			0.3				0.8
1(Lowest)	21/10	1			12/18	1		
2,3 and 4	27/68	0.67	0.35-1.28		36/59	1.19	0.58-2.43	
Minimum Mast Cell Count								
1(Lowest)	12/20	1			12/19	1		
2	7/26	0.66	0.26-1.68		14/22	1.50	0.67-3.37	
3	8/24	0.52	0.22-1.26		14/16	1.08	0.46-2.54	
4(Highest)	13/16	0.97	0.46-2.06		8/20	1.11	0.44-2.85	
	P trend			0.7				0.9
1(Lowest)	12/20	1			12/19	1		
2,3 and 4	28/66	0.72	0.39-1.35		36/58	1.24	0.61-2.51	
AA								
Minimum Mast Cell Density								
1(Lowest)	15/19	1			10/18	1		
2	11/13	0.98	0.35-2.79		12/18	0.98	0.36-2.66	
3	9/17	1.20	0.41-3.53		14/11	1.13	0.39-3.32	
4(Highest)	5/9	0.96	0.30-3.11		3/12	0.55	0.13-2.39	
	P trend			0.9				0.6
1(Lowest)	15/19	1			10/18	1		

2,3 and 4	25/39	1.04	0.44-2.45		29/41	0.94	0.38-2.34	
Minimum Mast Cell Count								
1(Lowest)	14/16	1			12/15	1		
2	12/15	0.74	0.29-1.91		8/20	0.61	0.19-1.95	
3	8/18	1.15	0.35-3.80		16/14	1.14	0.46-2.85	
4(Highest)	6/9	0.87	0.30-2.49		3/10	0.52	0.12-2.20	
	P trend			0.9				0.8
1(Lowest)	14/16	1			12/15	1		
2,3 and 4	26/42	0.85	0.38-1.86		27/44	0.86	0.37-2.04	

*, adjusted for age, race (only for all), grade, stage, PSA and BMI.

Table 3.5. Adjusted Hazard Ratio* (HR) of prostate cancer metastasis by race and mast cell measurements, 338 Case Race Disparity TMA.

All	Tumor Tissue				Benign Tissue			
Minimum Mast Cell Density	Case/Control	HR	95% CI	p	Case/Control	HR	95% CI	p
1(Lowest)	15/50	1			8/50	1		
2	8/47	1.43	0.48-4.23		9/53	0.79	0.26-2.43	
3	6/53	0.73	0.23-2.31		8/49	0.72	0.22-2.40	
4(Highest)	1/44	0.14	0.02-1.06		5/41	0.87	0.25-3.05	
	P trend			0.05				0.8
1(Lowest)	15/50	1			8/50	1		
2,3 and 4	15/144	0.65	0.28-1.47		11/143	0.79	0.30-2.07	
Minimum Mast Cell Count								
1(Lowest)	15/47	1			10/48	1		
2	8/52	0.86	0.30-2.44		8/56	0.92	0.31-2.74	

3	6/52	0.72	0.22-2.37		8/52	0.79	0.27-2.28	
4(Highest)	1/43	0.11	0.01-0.85		4/37	0.71	0.20-2.52	
	P trend			0.02				0.5
1(Lowest)	15/47	1			10/48	1		
2,3 and 4	15/147	0.51	0.22-1.15		20/145	0.81	0.34-1.94	
CA								
Minimum Mast Cell Density								
1(Lowest)	10/21	1			4/26	1		
2	2/29	0.96	0.19-4.96		4/28	1.18	0.25-5.57	
3	4/29	0.90	0.26-3.17		5/27	1.32	0.27-6.48	
4(Highest)	1/30	0.18	0.02-1.52		4/27	1.54	0.32-7.30	
	P trend			0.2				0.6
1(Lowest)	10/21	1			4/26	1		
2,3 and 4	7/88	0.67	0.35-1.28		13/82	1.34	0.36-5.02	
Minimum Mast Cell Count								
1(Lowest)	10/22	1			4/27	1		
2	2/31	0.64	0.12-3.32		5/31	1.76	0.42-7.44	
3	4/28	0.90	0.25-3.24		5/25	1.82	0.40-8.27	
4(Highest)	1/28	0.15	0.02-1.32		3/25	1.30	0.25-6.73	
	P trend			0.1				0.7
1(Lowest)	10/22	1			4/27	1		
2,3 and 4	7/87	0.50	0.18-1.41		13/81	1.64	0.46-5.78	
AA								
Minimum Mast Cell Density								

1(Lowest)	5/29	1			4/24	1	
2	6/18	3.74	0.42-33.48		5/25	1.45	0.09-23.60
3	2/24	-			3/22	0.21	0.01-5.47
4(Highest)	0/14	-			1/14	0.75	0.03-17.63
	P trend			0.1			0.4
1(Lowest)	5/29	1			4/24	1	
2,3 and 4	8/56	1.01	0.12-8.41		9/61	0.62	0.07-5.86
Minimum Mast Cell Count							
1(Lowest)	5/25	1			6/21	1	
2	6/21	1.26	0.17-9.61		3/25	0.25	0.02-3.20
3	2/24	-			3/27	0.11	0.004-2.80
4(Highest)	0/15	-			1/12	0.16	0.01-4.68
	P trend			0.1			0.2
1(Lowest)	5/25	1			6/21	1	
2,3 and 4	8/60	0.42	0.06-3.04		7/64	0.18	0.02-1.81

*, adjusted for age, race (only for all), grade, stage, PSA and BMI.
Statistically significant values are highlighted in red.

Due to the unexpected finding that revealed a relationship between low mast cell numbers and the development of metastases after radical prostatectomy in the present study, we next determined whether prostate cancer metastases contained high or low densities of intratumoral mast cells. Rapid autopsy tissues from three prostate cancer patients were immunostained for tryptase and evaluated. Interestingly, for prostate, lung, and liver metastatic tissues, no intratumoral mast cells were seen infiltrating the tumor (Figure 3.1). However, infiltrating intratumoral mast cells were present in bone metastasis.

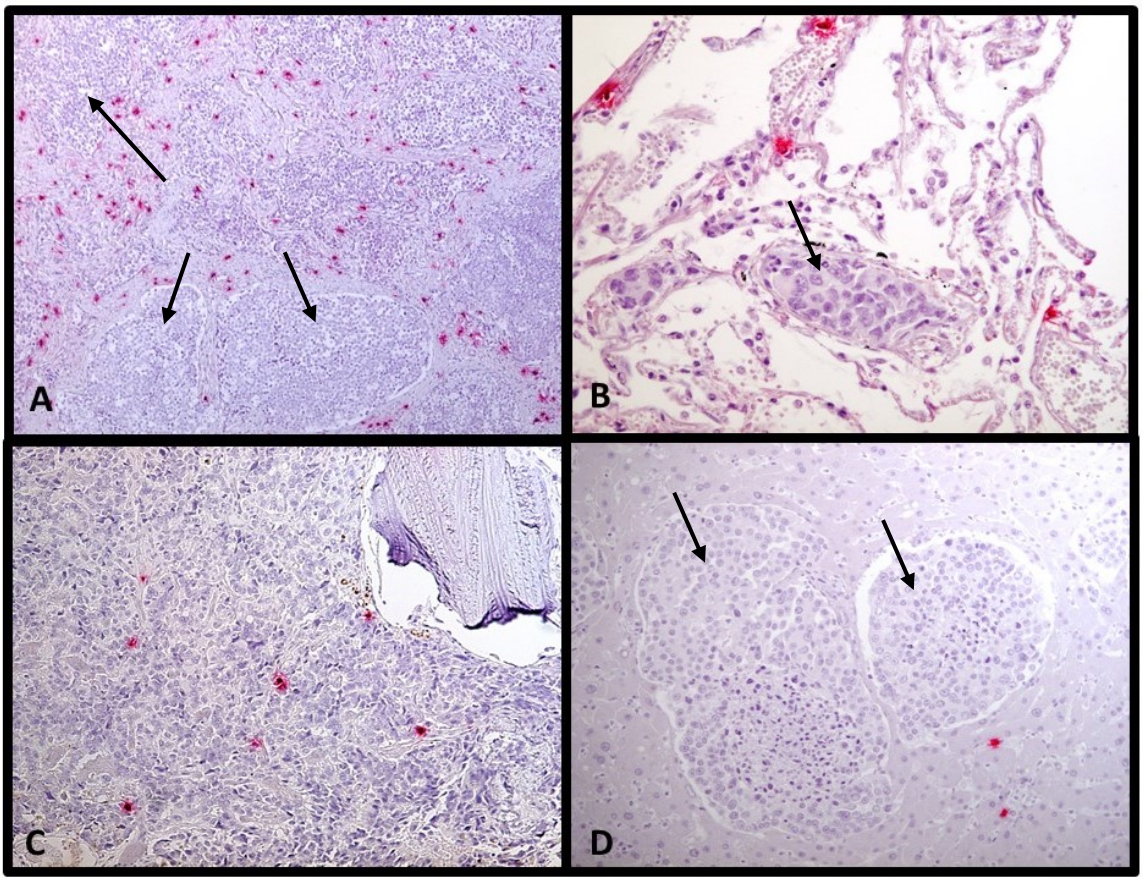


Figure 3.1. IHC for mast cell tryptase in a cohort of rapid autopsies from men with prostate cancer. A) Prostate cancer in a prostate at time of autopsy (40X). No intratumoral mast cells (red staining cells) are observed within tumor foci (arrows). **B**) Prostate cancer lung metastasis (arrow) at 200X with no intratumoral mast cells. **C**) Prostate cancer bone metastasis at 100X with infiltrating intratumoral mast cells. **D**) Prostate cancer liver metastasis (arrows) at 100X with no intratumoral mast cells.

3.5 Discussion and Conclusions

Given our previous results outlined in Chapter II, there is evidence to suggest that mast cells may be an indicator of cancer aggressiveness and/or recurrence. As African American men have been shown to be more likely to develop aggressive prostate cancer and to recur at a higher rate than Caucasian American men, as well as present with higher levels of inflammation, there was reason to study the role of mast cells race disparities in prostate cancer [86,87]. We aimed to address several questions to determine if mast cells are contributing factors in prostate cancer race disparities. First, do prostate tumor and benign tissues from AA men contain similar numbers and density of mast cells inside and outside the tumor compared to CA men? Secondly, do mast cells have the a similar relationship with risk of recurrence (PSA progression) in AA men as we previously observed; specifically, that low numbers of intratumoral mast cells correlate with higher risk of recurrence?

The first question was pursued by immunostaining and analysis of three cohorts represented on TMAs consisting of tumor and benign tissues from AA and CA men. Overall, the results showed no significant difference in mast cell number or density in tumor or benign tissues between AA and CA men, except when stratified by low versus higher Gleason grade (Tables 3.1-3.3). Tumors from AA men had significantly lower densities of mast cells compared to tumors of CA men, specifically in lower Gleason grade (Grade 6-7) tumors (Table 3.2, 3.3). The lack of difference in the higher Gleason scores may be due to the fact that the AA cases were matched to the CA cases on tumor grade and stage. Therefore, if mast cell density is truly associated with risk of recurrence in AA as well as CA men, then the mast cell densities would be similar. In retrospect, it may be better to determine whether AA men have lower densities of mast cells compared to CA men in a

cohort that is not closely matched on clinical parameters. Unfortunately, we did not have access to such a cohort, and performed our analysis in the available TMAs.

The second question was whether mast cells have the same relationship with PSA progression in AA men as they do in CA men. This question was studied in a high risk AA TMA with associated outcome data. When mast cell density was studied in relation with risk of recurrence, the results, though not significant, followed a similar trend as in Chapter II (Table 3.4). Interestingly, when we assessed the association of mast cell number and density with the development of metastases, we observed that low numbers of mast cells were significantly correlated with the development of metastases (Table 3.5). These results were also intriguing in light of our immunostaining results in metastatic lesions (Figure 3.1), where in all tissues except for bone, mast cells were not seen to be infiltrating the tumor.

As AA men are known to be more likely to develop metastasis and often present with an aggressive prostate cancer, it is possible that the AA men with lower grade tumors (Gleason 7) at radical prostatectomy will eventually go on to experience a biochemical progression and/or metastasis, thereby explaining the difference. This should certainly be the focus of future follow up studies.

IV. MAST CELL PHENOTYPING IN PROSTATE CANCER VERSUS BENIGN PROSTATE TISSUES

4.1 Abstract

Our previous studies suggest that mast cells have a differential relationship with prostate cancer prognosis depending on whether they are present inside or outside the tumor.

As such, in the present study, we questioned whether mast cells or their immediate microenvironments were phenotypically different based on whether they were present within cancerous or benign regions of the prostate. As an initial approach, we used IF assays to determine the mast cell subtype ratios in tumor and benign tissues via the expression of chymase, a protease that has been traditionally used to differentiate between the two classical subtypes of mast cells, MCtc, and MCt. We further used a toluidine blue stain and LCM technique to enrich for mast cells and their nearest interacting cells from frozen tumor and benign prostate tissues. RNAseq was performed for expression analysis of the samples, and multiple genes of interest from this dataset were chosen for further validation. An isoform of C-kit, C-kit Variant 1, was found to be more highly expressed in mast cells located in benign tissue versus tumor-infiltrating mast cells, a relationship corroborated by PCR-based techniques. C-X-C chemokine receptor type 4 (CXCR4) and Transcription Factor Binding To IGHM Enhancer 3 (TFE3), both of which are associated with mast cell activation, were found to be more highly expressed in tumor-associated than benign prostate tissue mast cells. This expression pattern was verified via *in situ* hybridization techniques, which indicated that expression of CXCR4 and TFE3 are increased in multiple cell types within tumor and inflamed regions. These results indicate that prostate tumor-infiltrating mast cells are phenotypically different from benign tissues mast cells and support the hypothesis that mast cells could play varying roles in intratumoral compared to extratumoral regions of the prostate.

4.2 Introduction

As seen in both Chapters II and III, our studies suggests mast cells have a differential relationship with prostate cancer prognosis depending on whether they are found within or outside the tumor. Given this differential relationship between mast cells number/density and biochemical recurrence and/or metastases based on mast cell geographical location, we aimed to determine whether mast cells or their immediate microenvironment are biologically different inside versus outside the tumor as a potential factor in their dichotomous relationship.

As discussed in Chapter I, mast cells originate in the bone marrow and undergo their final differentiation in their final tissue [1,7]. This differentiation can be between the two classical subtypes, MCtc and MCt, or between more subtle differences between protease and effector molecular expression profiles [3,7,26,27]. In addition, there is mounting evidence that mast cell profiles within the microenvironment are not final, either by mast cell plasticity or by recruitment of mast cell precursors that eventually develop into different subtypes based on signals in the microenvironment [1,28,29,121]. In one study, mast cell markers were studied in patients with and without severe asthma in order to analyze changes in mast cell subtype [121]. Endobronchial biopsies, epithelial brushings, and bronchoalveolar lavage samples were analyzed with IHC and qPCR techniques for total mast cell number and mast cell subtype to obtain a MCtc/total mast cell (Mctot) ratio[121]. Results showed that MCtc/MCtot was increased in severe asthma compared to mild asthma/no inhaled corticosteroid treatment group [121]. These results, in addition to other studies, suggest that the mast cell profile in the disease microenvironment can shift [118,121]. As such, there is ample reason to suggest that

expression profiles of mast cells within or outside the tumor would be different. In addition, it stands to reason that interactions between those mast cells and other cells in the microenvironment would also change.

Mast cells interact with many cells in the tissue microenvironment, including inflammatory cells, fibroblasts, and epithelial cells. Mast cells are known to interact with neutrophils, B cells, T cells, Tregs, and NK cells [1,122]. Mast cells can also produce cytokines promoting angiogenesis and cancer cell proliferation [27,39-41,113]. Finally, mast cells are well known to interact with fibroblasts, where fibroblasts produce a number of factors capable of influencing, regulating, and recruiting mast cell activity [106]. As such, we hypothesized the immediate microenvironments and cells directly interacting with mast cells within and outside the tumor may differ.

In order to explore the biology behind the dichotomous relationship between tumor and benign tissue mast cells, we identified and isolated matched tumor-infiltrating and benign tissue mast cells from frozen prostate tissues via LCM and conducted RNAseq experiments. The results of these studies indicated that the gene expression profile of tumor-infiltrating mast cells is vastly different from that of benign tissue mast cells. Four markers of interest were chosen for validation via quantitative PCR (qPCR) and RNA in-situ hybridization techniques, namely C-kit variants 1 and 2, CXCR4, and TFE3 variant 2, all of which are associated with mast cell activation.

4.3 Methods

Tryptase-Chymase double IF. TMAs were treated with HTTR antigen retrieval followed by incubation with anti-chymase antibody (Abcam ab18617 mouse-raised, 1:2000) and anti-

tryptase antibody (Abcam Ab2378 rabbit-raised, 1:2000). Antigen detection was accomplished with goat-ant-rabbit Cy3 secondary and chicken-anti-mouse Cy5 secondary (A11036 and A21463 respectively, both 1:100), as well as treatment with DAPI for nuclear visualization.

Toluidine blue staining and LCM. Frozen tumor and matched benign prostate tissues from 4 radical prostatectomy specimens (all Gleason grade 9) were cut onto Leica PEN-membrane 4,0um Frame slides, fixed in 90% ethanol in DEPC, and toluidine blue stained with 0.1% toluidine blue (Sigma) in DEPC treated with 0.4U/uL Protector RNase Inhibitor (ref. 03335402001). LCM was performed on the Leica LMD 7000 Microscope.

RNA extraction and RNAseq. RNA extraction was accomplished with Qiagen RNeasy Micro Kit. Tumor and benign mast cell RNA samples were pooled for each case (resulting in one pooled tumor sample and one pooled benign sample), RNA quality was assessed using a Bioanalyzer, and RNA sequencing (RNAseq) was performed at the Sidney Kimmel Comprehensive Cancer Center Next Generation Sequencing Core Facility.

C-kit variant 1 qPCR and RT-PCR. qPCR was performed with C-kit variant 1 forward primer 5'-CAACAAAGAGCAAATCCATCCC-3' and reverse primer 5'-CATCACAATAATGCACATCATGCC-3' with iQ SYBR Green supermix (Cat. No. 1708882). The PCR program was as follows: 94ºC for 3 min followed by 40 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 30 seconds. RT-PCR and KIT variant genotyping was performed with C-kit forward primer 5'-CAGCAAGGAGTGAACAG-3'

and reverse primer 5'-GGGGGATCCGATGTGGGAAGACTTCT-3'. The program was as follows: 94ºC for 3 min followed by 40 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 30 seconds, finished with 72ºC. Products were run on a 4% gel.

Control cell plugs for Kit V1 and V2, CXCR4, TFE3. Control cell plugs were made by transfection of PC3 cells with C-kit Variant 1 human ORF cDNA clone (Origene NM_000222), C-kit Variant 2 human ORF cDNA clone (Origene NM_001093772), CXCR4 human ORF cDNA clone (Origene NM_003467), TFE3 variant 2 human ORF cDNA clone (Origene NM_001282142). Transfection was accomplished with Lipofectamine 2000 (Life Technologies L0000-001). Cells were spun down, fixed in 10% formulin at least 48hrs, and paraffin embedded.

RNA in situ hybridization (RISH). 1zz probes were designed by Advanced Cell Diagnostics (ACD) to be specific to c-kit variant 1 but not variant 2. The Basescope manual red kit was used to stain C-kit variant 1, variant 2, and no-plasmid control transfected cell plugs with variant 1 and 1zz PPIB probes. Staining of control plugs was also done with DNAse I (bovine Sigma Cat# D5319) or RNAse A (DNAse-free Affymetrix #78020Y) treatment steps to verify detection of RNA versus genomic DNA. The ACD RNAscope 2.5 manual brown kit was used to stain CXCR4 transfected and no plasmid control cell plugs with CXCR4 probe (ACD Cat# 310511), and TFE3-transfected and no-plasmid control cell plugs with TFE3 probe (Cat. No. 430461). ACD Dual RNAscope manual kit was used to double stain FFPE TMA 1170 for C-kit (Cat No. 606401)-CXCR4 (Cat No. 310511-C2), CXCR4 (Cat

No. 310511)-CD68 (Cat No. 560591-C2), and TFE3 (Cat. No. 430461)-C-kit (Cat No. 606401-C2).

4.4 Results

Mast cell subtyping using IF for tryptase and chymase. As an initial assessment of mast cell subtypes in relation to prostate cancer, we used a dual IF stain to analyze the 120 Case High Grade Race Disparity TMA set (see Chapter III) to determine if there was a difference in mast cell subtype ratios between tumor and benign tissues in relation to tumor grade or stage and in AA and CA men. As shown in Figure 4.1, MCt cells were visualized as tryptase only positive mast cells and MCtc cells were visualized as tryptase and chymase double positive mast cells.

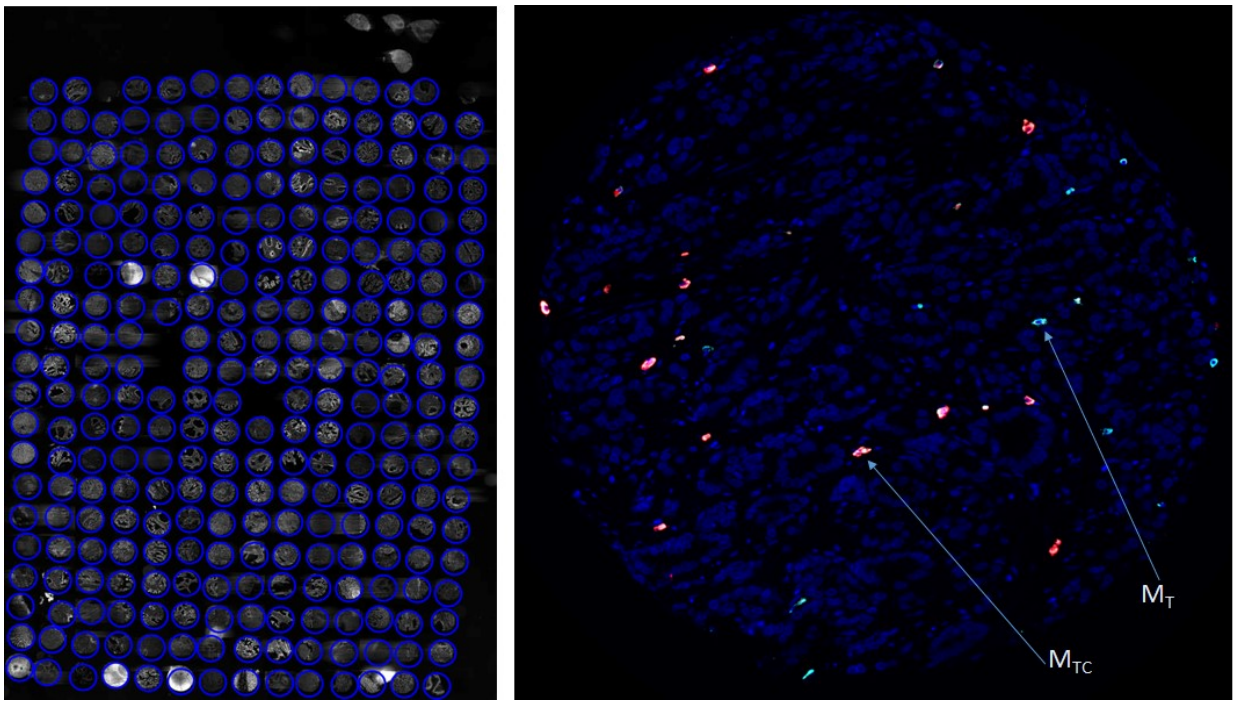
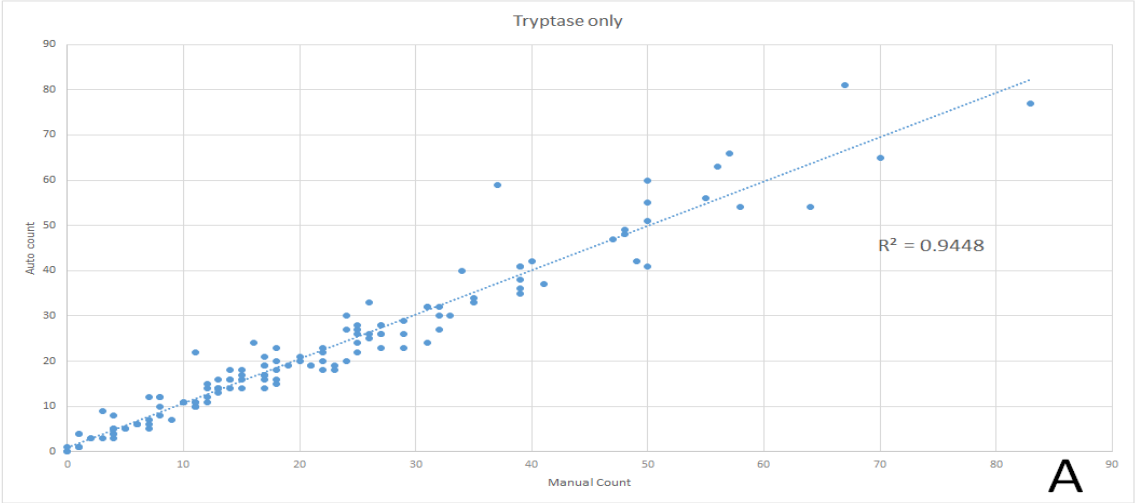


Figure 4.1 Dual IF for mast cell tryptase (green) and chymase (red). Left, TMA visualized by IF. Right, individual TMA spot showing mast cell subtypes visualized using dual IF, where total mast cells (MCtot) were all tryptase positive cells, MCtc were tryptase

and chymase double positive cells (arrow denoted as M_{TC}), and MCt were MCtot-MCtc tryptase only positive cells (arrow denoted as M_T).

We used automated image analysis software from TissueGnostics which is designed for image analysis of fluorescent images to identify and count the number of MCt and MCtc in each tissue spot represented across the TMAs. As with the PIP software that we used for chromogenic assessment of mast cells (see Chapters II and III), we began by comparing the automated counting to manual counting as the gold standard. We found a high correlation between manual counting and automated counting using the TissueGnostics software (R^2 = 0.94 for MCtot and 0.93 for MCtc, Figure 4.2).



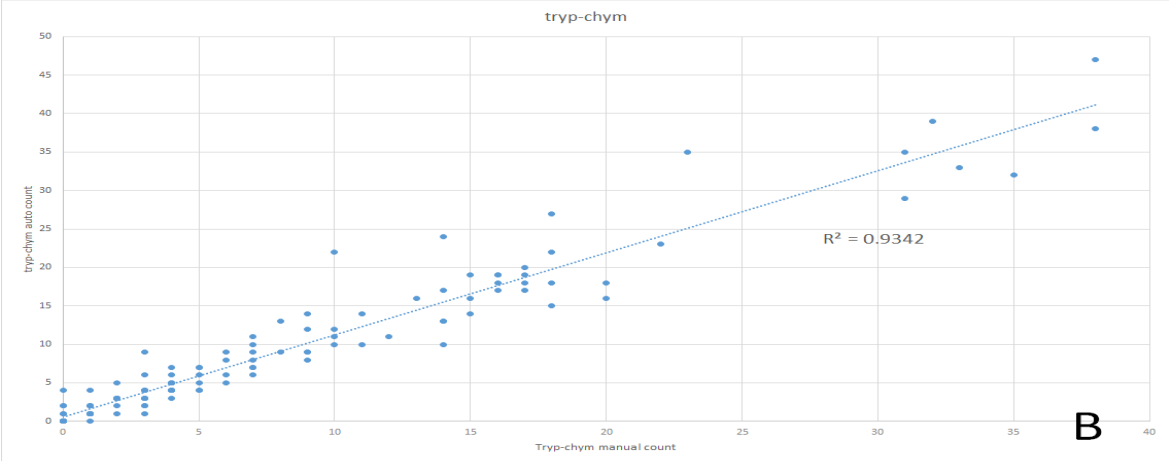


Figure 4.2. Comparison of manual vs TissueGnostics automated count of MCtot and MCtc in the 120 Case Race Disparity TMA. Comparison shows that TissueGnostics software provides an accurate count of both MCtot (A) and MCtc (B), with R^2 values of 0.94 and 0.93, respectively.

Interestingly, the results of these analyses indicated that there is a significantly greater proportion of MCt (tryptase-only mast cells) in prostate cancer compared to matched benign tissues and likewise a significantly greater proportion of MCtc (tryptase and chymase positive mast cells) in benign regions compared to cancer regions (Table 4.1). However, there were no differences found in MCt or MCtc mast cell subtypes between the AA and CA men in this cohort (Table 4.2).

Table 4.1. Paired comparison of mast cell subtype ratios between tumor and benign tissues in the 120 Case Race Disparity TMA set.

Ratio* of Tryptase Only	N	Minimum	Maximum	Mean	Median	STD***
Tumor	117	0.41	0.90	0.68	0.69	0.15
Benign	117	0.27	0.92	0.58	0.58	0.19
p**		0.0002	0.7	0.02	0.03	<.0001
Ratio of Tryptase_Chymase						
Tumor	117	0.10	0.59	0.32	0.31	0.15
Benign	117	0.08	0.73	0.42	0.42	0.19
p**		0.7	0.0002	0.02	0.03	0.05

* Ratio=subtype mast cell count/total mast cell count
** p: Paired comparison using Wilcoxon signed-rank test
*** N=111

Table 4.2. Summary of mast cell subtype measurements in the 120 Case Race Disparity TMA set.

Ratio* of Tryptase_Chymase						
Tumor Tissue	N	Minimum	Maximum	Mean	Median	STD
CA	59	0.1	0.6	0.3	0.3	0.1
AA	59	0.04	0.6	0.3	0.3	0.2
p**		0.7	0.8	0.9	0.9	0.9
OR*** (95% CI)		1.2 (0.5-2.5)	1.3 (0.5-3.0)	0.7 (0.3-1.7)	1.0 (0.4-2.6)	0.9 (0.4-2.3)
Normal Tissue						

CA	60	0.05	0.7	0.4	0.3	0.2
AA	60	0.1	0.8	0.4	0.4	0.2
p		0.3	0.3	0.2	0.1	0.5
OR (95% CI)		0.9 (0.4-2.1)	2.1 (0.9-4.8)	1.3 (0.5-3.2)	1.4 (0.6-3.4)	0.7 (0.3-1.8)
Ratio of Tryptase Only						
Tumor Tissue						
CA	59	0.4	0.9	0.7	0.7	0.1
AA	59	0.4	1.0	0.7	0.7	0.2
p		0.8	0.7	0.7	0.9	0.9
OR (95% CI)		1.1 (0.5-2.6)	-	0.9 (0.3-2.2)	1.1 (0.5-2.8)	0.9 (0.4-2.3)
Normal Tissue						
CA	60	0.3	1.0	0.6	0.7	0.2
AA	60	0.2	0.9	0.6	0.6	0.2
p		0.3	0.3	0.2	0.1	0.5
OR (95% CI)		2.1 (0.9-4.8)	-	0.7 (0.3-1.8)	0.6 (0.2-1.5)	0.7 (0.3-1.8)

* Ratio=subtype mast cell count/total mast cell count
** p: From Wilcoxon signed-rank test
*** OR (odds ratio, AA vs. CA): For minimum, odds of falling below first quartile of the respective mast cell measure in CA men. For the other measures, odds of falling above third quartile of the respective mast cell measure in CA men. Logistic regression model is adjusted for age, stage, grade and surgical margin status.

Gene expression analysis of tumor-infiltrating and benign tissue mast cells. Toluidine blue staining of frozen tissues both allowed for visualization of mast cells in purple against the blue counterstain (Figure 4.3) as well as preservation of the mRNA from the cells collected. Separately, we collected mast cells from tumor and benign regions from four cases, extracted RNA, and performed RNAseq. The RNAseq data identified reads from 9,041 different genes expressed in these samples. Of these genes, 1,457 were at least 3-fold greater in transcripts per kilobase million (TPM) in tumor mast cells versus benign and 2,561 genes were 3-fold greater in TPM in benign tissue mast cells versus tumor. Importantly, there was a

strong representation of mast cell related genes (Table 4.3), supporting the conclusion that mast cell enrichment was achieved. Important genes that identify mast cells such as tryptase subunits alpha/beta1, beta 2, and gamma 1, as well as C-kit, chymase, VWASA, and GATA2, were found to have no significant differences in expression between the two groups. Other genes, however, were found to be different, with CD44 being more highly expressed in mast cells isolated from benign tissues, and CXCR4 and TFE3 being more highly expressed in mast cells isolated from tumor tissues.

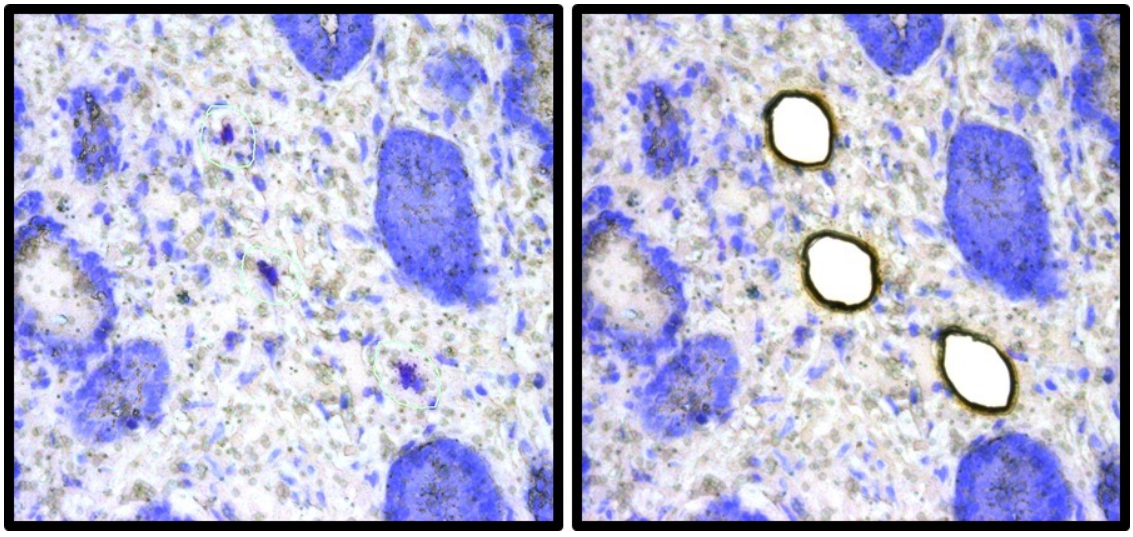


Figure 4.3. Image of toluidine blue staining and LCM for enrichment of mast cells and the immediate microenvironment. Mast cells and nearby nuclei were successfully isolated via LCM.

Genetic isoforms were also analyzed for differences in expression between tumor and benign tissue mast cells. One gene of particular interest was C-kit, and its' isoforms 1 and 2. Although overall C-kit expression was the same in tumor and benign (Table 4.3), C-kit Variant 1 was more highly expressed in benign tissue mast cells, while C-kit variant 2 was more highly expressed in tumor tissue mast cells (Table 4.4).

Table 4.3. Select genes identified in tumor and benign tissue mast cells via RNAseq. There were a high number of mast cell-related genes identified in the RNAseq data, validating that mast cells were successfully enriched via LCM. Genes highlighted in green were more highly expressed (> 3 fold) in tumor mast cells, yellow were similarly expressed in tumor and benign, and red were more highly expressed (> 3 fold) in benign. Two genes that were highly differentially expressed between tumor and benign tissue mast cells are TFE3 and CXCR4.

Gene	prob	log2(B9/cancer)	TPM Benign	TPM Cancer	Notes
TFE3	1.00	-15.23	0.00	26432.08	Potential regulator of mast cell functions (PMID 22360977)
RSBN1	1.00	-13.63	2.14	35950.60	
CXCR4	1.00	-12.48	2.14	16149.36	Expressed within the mast cell lineage and its ligand SDF-1alpha/CXCL12 acts as a mast cell chemotaxis
ARHGAP5	1.00	-12.33	10.70	58521.43	Upregulated in mast cells in response to infection with live streptococci (PMID 19933827)
ICMT	1.00	-11.54	11.00	34751.87	
MMP24	1.00	-9.55	0.00	519.65	Involved in cell-cell interactions between nociceptive neurites and mast cells (PMID 19805319)
STAT3	1.00	-8.95	2.15	1402.82	
KMT2A	1.00	-8.78	251.99	111413.54	mutated in systemic mastocytosis (PMID 25305106)
CXCL12	1.00	-8.37	0.00	229.90	
PTPN11	1.00	-7.389	0.54	205.6	Positively regulates KIT, required for mast cell survival and homeostasis, promotes chemotaxis (PMC4383135)
CD74	1.00	-6.96	28.36	3620.46	Complexes with CXCR4, receptor for MIF (PMID 19665027)
TPSD1	1.00	-6.92	3.75	535.27	Tryptase delta 1
VWA5A	0.64	-3.56	3837.21	45255.96	Mast cell surface antigen 1
FCER1A	0.70	-3.06	272.86	2284.03	IgE receptor subunit alpha
TPSAB1	0.17	-1.52	3138.91	8977.96	Tryptase alpha/beta 1
MALAT1	0.03	-0.74	169451.36	282588.33	
HPGDS	0.14	-0.74	3371.14	5618.57	Catalyzes conversion of PGH2 to PGD2 and plays a role in the production of prostanoids in mast cells
CPA3	0.09	-0.65	10456.93	16433.19	Mast cell carboxypeptidase A
HRH4	0.64	-0.62	3.41	5.61	Histamine receptor H4
KIT	0.11	-0.52	6254.21	8950.15	Mast/stem cell growth factor receptor
TGFBR2	0.20	-0.44	929.31	1263.51	Receptor for LRG1, may be a key regulatory factor of allergic responses (PMID 27378305)
LRG1	0.34	-0.43	155.40	210.12	Ligand for TGFBR2, may be a key regulatory factor of allergic responses (PMID 27378305)
TPSB2	0.20	0.56	1746.96	1181.14	Tryptase beta 2
HDC	0.15	1.25	11114.64	4661.55	Histidine decarboxylase
CMA1	0.43	1.51	399.12	140.18	Chymase
STX3	0.32	1.84	2175.62	607.46	Controls distinct essential steps in mast cell degranulation (PMID 24323579)
TPSG1	0.76	2.32	2.79	0.00	Tryptase gamma 1
IL1RL1	0.44	2.90	4577.46	613.07	IL1RL1/ST2 gene encodes receptor for IL-33 (modulator of Th2) in mast cells (PMID 22865859)
GATA2	0.50	3.32	6476.36	648.58	Critical for mast cell differentiation and maintenance (PMID 25801432)
HRH1	0.83	3.35	82.72	7.48	Histamine receptor H1
CD44	0.79	5.29	18053.72	459.80	Involved in mast cell proliferation and terminal differentiation (PMID 19204665)
DUSP1	0.93	6.34	7145.33	87.85	May modulate mast cell activation and allergic reaction (PMID 17636038)
CERK	0.98	7.18	100.12	0.00	Mediator of calcium-dependent degranulation in mast cells (PMID 14769792)
LTB4R	0.99	7.47	452.62	1.87	Recruitment of mast cells in response to LTB4 production in areas of inflammation (PMID 16920986)
IL15	0.99	8.26	1358.77	3.74	Controls mast cell survival and homeostasis (PMID 19632221), suppresses chymase activities (PMID 17643110)
MRGPRX2	0.99	8.55	256.81	0.00	Controls secretagogue-induced histamine release (PMID 25517090)
IGF1	0.99	8.80	9499.62	20.65	Promotes mast cell survival (PMID 17046573)
FCER1G	1.00	11.33	1783.74	0.00	IgE receptor subunit gamma

prob – probability of differential expression

Table 4.4. C-kit variants 1 and 2 show differential expression in tumor compared to benign tissue mast cells. Although overall C-kit (KIT) expression was roughly equivalent between tumor and benign, there was differential expression of C-kit variants, with Variant 1 showing higher expression in benign, and Variant 2 showing higher expression in tumor.

gene_ name	transcript_ID	probability_ diff_expression	log2(Benign /Tumor)	Benign Mean	Tumor Mean	notes
KIT	NM_000222	1	13.473	6354.4033	0.0355277	Transcript variant 1 (NM_000222.2)
KIT	NM_001093772	0.989	-5.229	226.25739	8506.1435	Transcript variant 2, uses alternate in-frame splice site in central coding region (NM_001093772.1)

We chose to pursue validation of differential expression of C-kit variant 1, CXCR4, and TFE3 in tumor and benign tissue mast cells, as well as determine the localization of the expression of these genes in the tumor microenvironment.

C-kit variant 1 expression analysis. PCR primers designed against the C-kit variant 1 region that is absent in variant 2 were used to perform reverse transcription qPCR on mRNA collected from prostate tumor and matched benign tissues. The RNA samples were from harvested frozen prostate tissues (not LCM isolated mast cells) from a range of Gleason score tumors and their adjacent benign prostate tissues. In all cases, expression of C-kit variant 1 was higher in RNA from benign tissues than in the matched tumor tissue RNA (Figure 4.4). In addition, primers designed to detect both C-kit variant 1 and variant 2 mRNA were used to perform reverse transcription PCR (RT-PCR) and the products run on a 4% agarose gel to detect both variants simultaneously in the same RNA samples (Figure 4.5). These primers were found to preferentially amplify variant 2 over variant 1 when using equal amounts of plasmid DNA containing each C-kit variant (data not shown), so the data were not quantifiable. This experiment did, however, serve to confirm the presence of both gene isoforms in both tumor and benign tissue RNA samples in this sample set.

Finally, we designed an *in situ* hybridization probe that was specific to C-kit variant 1 using the BaseScope assay from Advanced Cell Diagnostics. We validated the specificity of the probe using PC3 cells transfected with expression vectors containing C-kit variant 1 or 2 (Figure 4.6). An analysis of a small TMA set containing tumor and matched benign tissues

from 5 radical prostatectomy specimens showed that C-kit variant 1 expressing mast cells were often found in benign regions of the prostate as opposed to cancer (Figure 4.6).

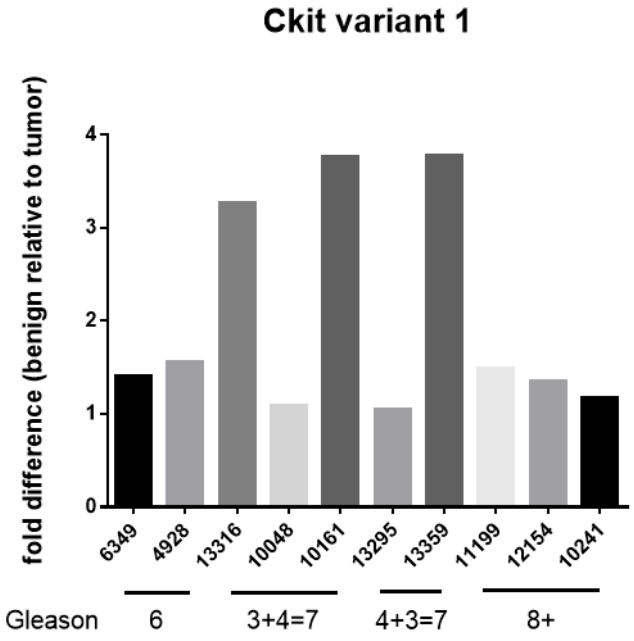


Figure 4.4. qPCR for C-kit Variant 1. Across Gleason scores, benign tissue had higher expression of C-kit variant 1 mRNA than matched tumor tissue.

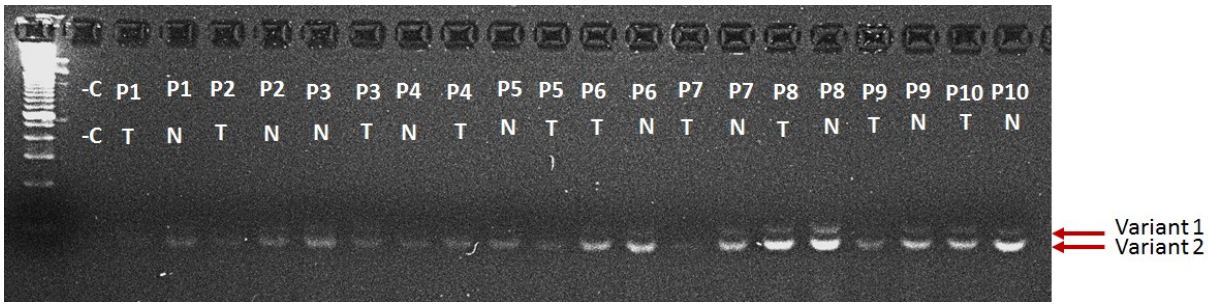


Figure 4.5. RT-PCR verification of the presence of both C-kit variants in tumor and matched benign prostate mRNA. The PCR primers were found to preferentially amplify Variant 2, so the data is not quantitative and direct comparison of expression levels cannot be accomplished with this method.

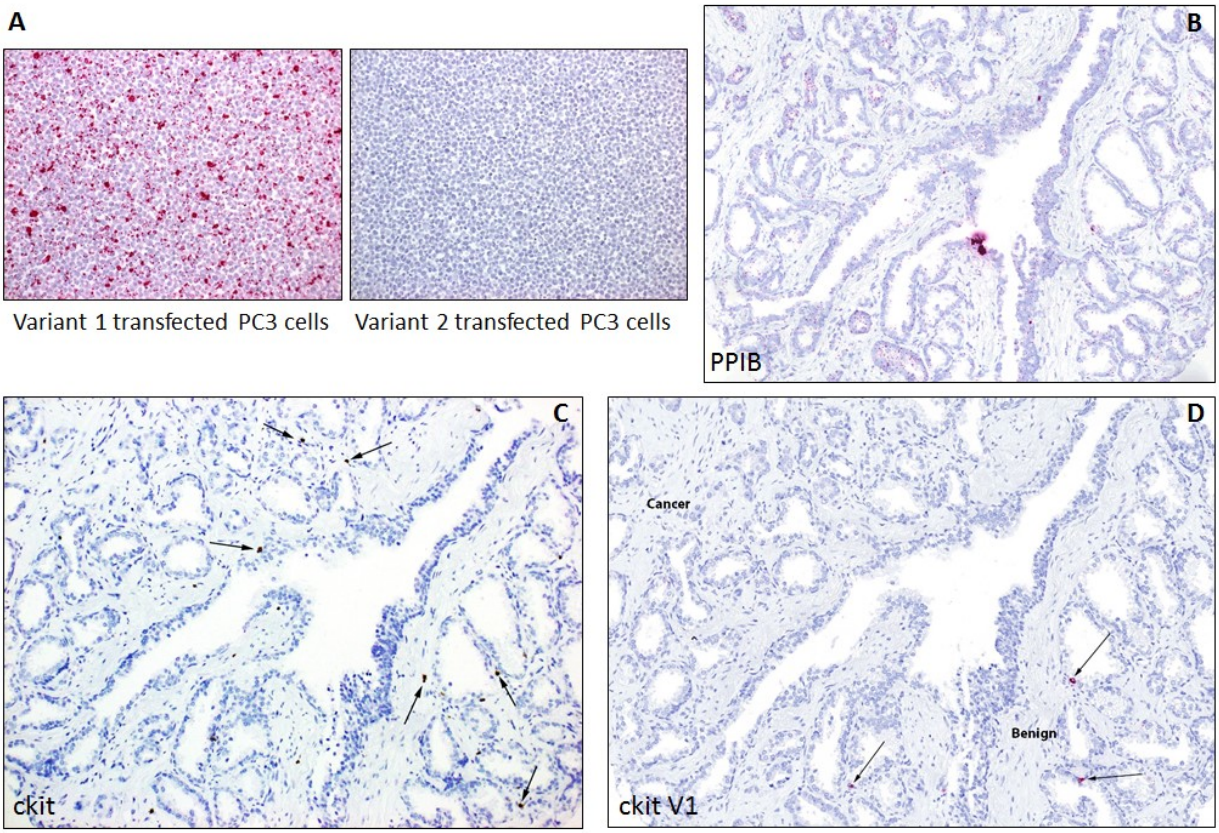


Figure 4.6. Visualization of c-kit variant 1 using RISH. **A**) Validation of the variant 1 (V1) probe set in variant 1 or variant 2 transfected PC3 cells. **B**) Basescope RISH assay for 1zz PPIB (red chromogen, positive control, **C**), RISH for c-kit (all variants, brown chromogen, and **D**) Basescope RISH for c-kit variant 1 (red chromogen). C-kit positive mast cells (arrows) are present in all regions as seen in panel C, but c-kit V1 expressing mast cells (arrows) are only present in benign regions in panel D.

Visualization of CXCR4 expression using RISH. RNAscope probes designed against CXCR4 mRNA were validated via the transfection of CXCR4 plasmid into PC3 cells and treatment with the RNAscope 2.5 brown kit. PPIB RNA quality positive controls showed high quality RNA, and the CXCR4 probe was specific to the cell plug transfected with the CXCR4 expression vector (Figure 4.7). CXCR4 RISH on prostate tissues showed limited CXCR4 expression in normal appearing prostate tissues and much higher levels of CXCR4 expression in areas with cancer and areas with inflammation (Figure 4.8). The positive

CXCR4 signals were primarily present in the stromal compartment and appeared to be in inflammatory cells. Dual *in situ* hybridization was performed to elucidate if CXCR4 expression was found mainly in mast cells or in other inflammatory cells. The results showed that, although some positive mast cells could be seen (Figure 4.9), a large number of the positive inflammatory cells were likely lymphocytes, many of which could be seen in close proximity to mast cells. Many CXCR4 positive macrophages were also identifiable in the tumor microenvironment (Figure 4.9).

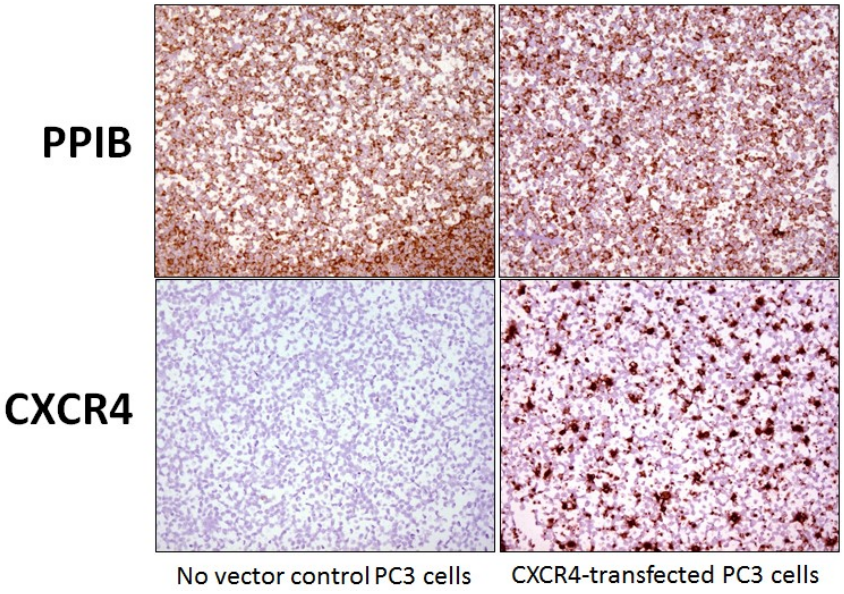


Figure 4.7. PPIB (positive control) and CXCR4 RISH on transfected cell plug controls. The CXCR4 RISH probe was specific to the cell plug transfected with CXCR4 plasmid.

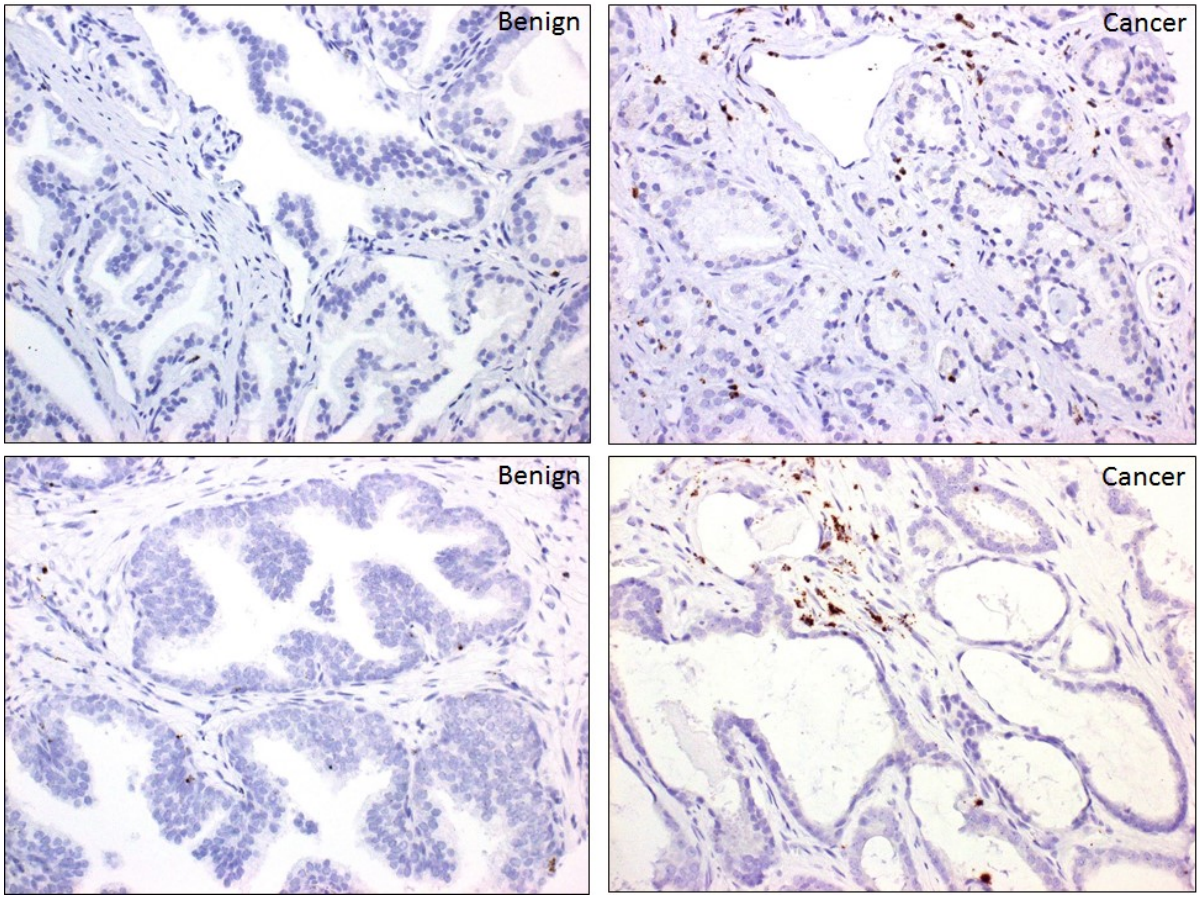


Figure 4.8. CXCR4 in situ hybridization in prostate tissue. CXCR4 (brown staining) appeared to be higher in cancer regions and areas of inflammation.

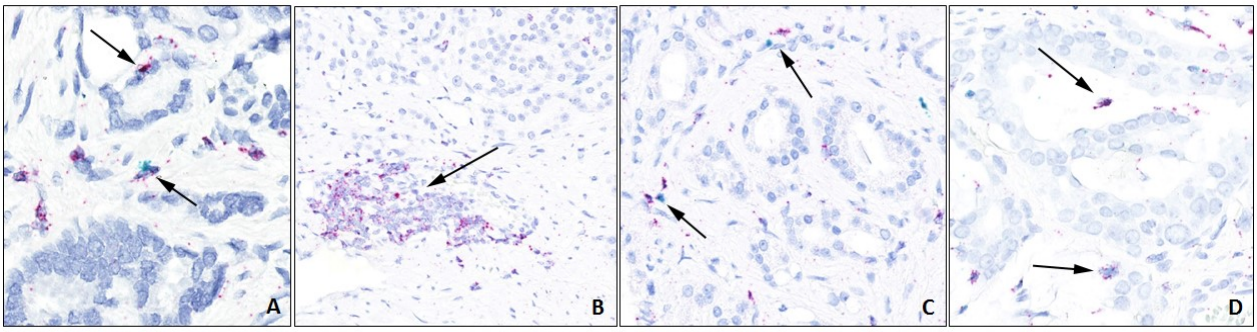


Figure 4.9. Dual *in situ* hybridization demonstrating CXCR4+ mast cells, CXCR4+ lymphocytes, mast cells in close proximity to CXCR4+ lymphocytes, and CXCR4+ macrophages. **A**) Presence of mast cells (arrows) double positive for C-kit (green) and CXCR4 (red). **B**) Presence of a cluster of lymphocytes (arrow) positive for CXCR4 (red). **C**)

C-kit+ mast cells (green) were seen to be in close proximity to CXCR4+ (red) inflammatory cells (arrows). **D**) Macrophages (arrows) double positive for CD68 (red) and CXCR4 (green).

Visualization of TFE3 expression using RISH. Visualization of TFE3 expression patterns via RNAscope RISH showed abundant expression of TFE3 in stromal and epithelial cells in both tumor and benign prostatic tissue. However, in benign tissue, mast cells were rarely observed in close proximity to TFE3 positive cells, while this was commonly observed between mast cells and TFE3 positive stromal cells in the tumor microenvironment (Figure 4.10). Occasional TFE3 and C-kit double positive mast cells were observed in tumor areas (Figure 4.10).

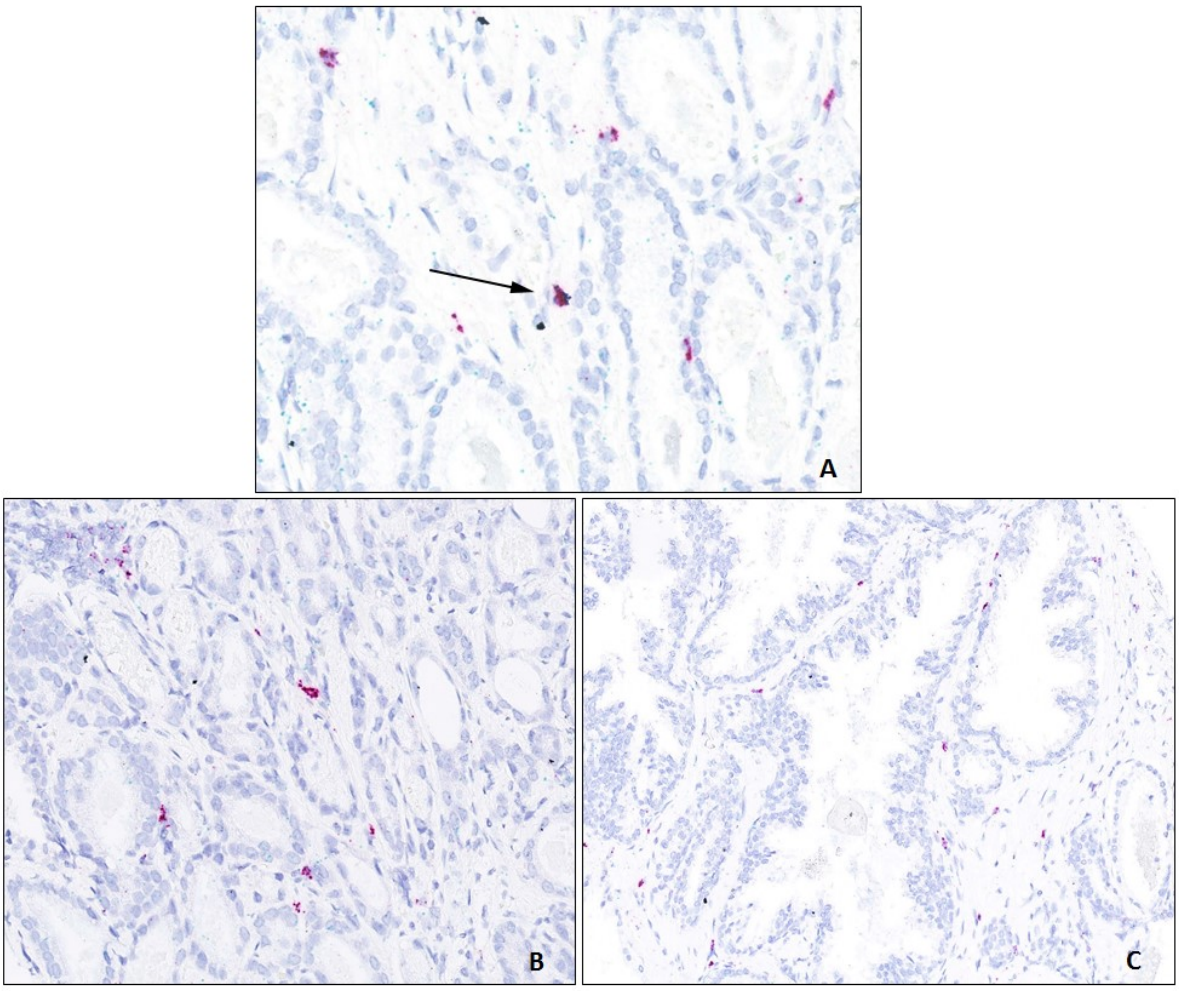


Figure 4.10. Dual *in situ* hybridization of TFE3 (green) and C-kit (red). TFE3 expression is present in both tumor and benign stromal and epithelial cells. However, **A**) TFE3 positive mast cells and mast cells associated with TFE3 positive cells occurred more frequently in **B**) tumor than in **C**) benign.

4.5 Discussion and conclusions

In previous studies, mast cells have been seen to have a differential relationship with risk of recurrence depending on whether they are intra- or extratumoral mast cells[115,120]. As such, there is evidence to suggest a potential difference in biology either within the mast cells themselves, or in the microenvironment interactions with those mast cells. Consequently, we sought to both enrich for mast cells inside or outside the tumor, as well as the immediate cellular microenvironment in direct contact with those mast cells. LCM proved to be an effective method to accomplish those goals, offering the additional benefits of minimal manipulation of the mast cells and the ability to be specific as to whether they were located in tumor or benign regions of the prostate.

Since mast cell expression profiles are potentially subject to the microenvironment, the use of other methods of enrichment, such as the use of antibodies for mast cell isolation, could have affected the results of the study. Importantly, isolating mast cells from homogenized tissue would have limited our ability to differentiate between intra- and extra-tumoral mast cells. Consequently, we chose a method that allowed direct visualization of the mast cells *in situ* with limited treatment and effects of sample manipulation on RNA expression. Toluidine blue stain and LCM proved to be an effective method for mast cell enrichment, as evidenced by the identification of numerous mast cell-related genes in the RNAseq data (Table 4.3).

LCM also allowed for the inclusion of immediately adjacent epithelial, stromal, and inflammatory cells. We found this to be a strength, as we were interested not only in the mast cell phenotypes, but their immediate interactions with their microenvironments. However, the strong presence of a large number of mast cell associated genes provides evidence that mast cells were enriched.

The mast cell-related genes identified by RNAseq included Tryptase, Chymase, C-kit, CXCR4, GATA2, TFE3, and GATA2 (see Table 4.3). Some of these genes showed no significant difference between tumor and benign mast cells, such as tryptase, chymase, and C-kit, which would be expected for mast cell marker genes. One gene of particular interest seen to be increased in benign prostate mast cells compared to tumor-infiltrating mast cells was C-kit variant 1, while several genes of interest were more highly expressed in tumor-infiltrating mast cells than benign, including CXCR4, and TFE3.

Although overall C-kit expression was roughly equivalent in tumor and benign, there was significant differences in the expression of C-kit variants in tumor compared to benign, where C-kit variant 1 was higher in benign tissue mast cells and variant 2 was higher in tumor-infiltrating mast cells. C-kit variant 1 is expressed in lower levels than variant 2, especially in the context of cancer, and variant 2 differs from variant 1 by the absence of 12 base pairs via an in-frame splice site variation [123,124]. Variant 2 has been shown in multiple studies to be associated with higher colony formation, lower contact inhibition, and higher tumorigenicity in mice when expressed in NIH3T3 fibroblasts [123]. Expression of variant 2 in early myeloid cells also results in higher and more rapid activation and internalization of SRC family kinases in response to SCF, as well as higher SCF-dependent growth and a stronger chemotactic response to SCF in-vitro compared to early myeloid cells expressing

variant 1 [125]. In addition, variant 2 has been associated with higher levels of granule formation, histamine content, and growth in mast cells as well as in faster response to SCF compared to variant 1, however variant 1 expression resulted in longer activation [126,127]. Finally, a higher serum variant 2/variant 1 ratio has also been associated with higher levels of neoplastic mast cells in mastocytosis [126]. Thus, mast cells expressing variant 2 are demonstrably more active than those expressing variant 1.

CXCR4 is a chemokine receptor for CXCL12 expressed by inflammatory cells including T cells, macrophages, and mast cells, and has been shown to play a role in mast cell recruitment [128-130]. The CXCL12-CXCR4 pathway in mast cells has been implicated to play a role in several cancers, including UV induced skin cancer, glioblastoma, and prostate cancer [106,131,132]. In addition, CXCR4 has been shown to be expressed by some prostate cancer cell lines including PC3 cells [133]. In addition, one study in an obesity and myc-induced mouse model found that the CXCR4-CXCL12 pathway was a driver in tumor migration and invasion, and blocking of CXCR4 sensitized the mice to chemotherapy [134]. Finally, meta-analysis of human data has found CXCR4 is significantly more highly expression in prostate tumor tissue than benign prostate tissue, and is associated with a higher prostate cancer stage, but not Gleason score [135]. This analysis also showed an association with lymph node involvement, bone metastasis, and poor prognosis. Interestingly, CXCR4 has also been highlighted in microarray studies as having higher expression in prostate cancer in African American men compared to Caucasian American men, potentially suggesting a role in the significant racial disparity found in prostate cancer [93,105]. Given our study, this may be an association worth further investigation.

TFE3 is a transcription factor previously reported to play a role in the functions of B and T cells, specifically in regulation of surface IgE and CD40L respectively [136]. TFE3 has also been demonstrated to play a role in mast cell degranulation, mediator secretion, and histamine release in response to an allergic trigger, all of which are hallmarks of mast cell activation [136]. In the context of cancer, TFE3 fusions have been identified in sarcomas (ASPS) and renal cell carcinoma, but not yet identified in prostatic adenocarcinoma [137].

These data supports the hypothesis that mast cells, and their immediate microenvironments, are biologically different in tumor compared to benign tissues. As prostate cancer is an extremely heterogeneous tumor environment, this may serve as an explanation as to why the literature is contradictory as to the relationship between mast cells and prostate cancer progression. Our findings also have interesting implications for prostate cancer treatment, as the mast cell populations appear to be diverse, and therefore may not be predictable in response to direct targeting or prostate cancer therapies. Additional studies are necessary to elucidate the exact pathways mast cells may be contributing to tumor progression and metastasis.

V. MAST CELLS IN ANIMAL MODELS OF PROSTATE CANCER

5.1 Abstract

Given our previous results suggesting a role for mast cells in PSA progression and metastasis, we next aimed to study whether mast cells may be playing a role in early invasion in prostate cancer. This is a question best asked in animal models, where prostate cancer development can be seen in different stages, as well as observed for direct cause and effect.

This was accomplished via two animal models; the rat PhIP model and the HiMyc-Wsh model. In the rat PhIP model, mast cells were quantified in the ventral prostates of rats that were treated with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and/or *E. coli* via transurethral infection and allowed to age another 32 weeks. In the HiMyc-Wsh mouse model, mast cell knock-out Wsh mice were crossed with the HiMyc mouse model to determine if the complete absence of mast cells would have an effect on the development of invasive carcinoma. In both models, mast cells were quantified via toluidine blue staining. In the PhIP model, mast cells were found to be increased in all rats treated with PhIP and/or *E. coli* even 32 weeks after treatment. In the HiMyc-Wsh model, the dorsal-lateral prostate was found to simultaneously have the most invasive carcinoma and the highest numbers of mast cells in wild-type and Wsh heterozygous mice. There was a decrease in invasive carcinoma in the mast cell knock-out Hi-Myc mice. Our findings support a role for mast cells in driving early invasion of prostate cancer.

5.2 Introduction

In our human studies of prostate cancer, evidence is mounting for a role for mast cells in predicting recurrence and metastasis. We next wanted to explore whether mast cells play a role in early invasion of prostate cancer, a question best addressed with animal models. Human radical prostatectomy specimens contain cancer that presumably developed many years prior to detection and treatment. We chose to pursue the question of early invasion via two rodent models, specifically the rat PhIP model and the HiMyc-Wsh mouse model.

There have been a number of studies exploring potential roles of mast cells in prostate cancer in rodent models, including one study in TRAMP mice by Pittoni *et al.* In their study,

Pittoni *et al* explored the role of mast cell MMP9 in early prostate tumor progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, arguing that lower grade prostate tumors would need mast cell-derived MMP9 for invasion, since well differentiated prostate tumor cells do not produce MMP9 [114]. The results did in fact suggest that mast cell MMP9 was necessary for early tumor invasion in mice. Immunohistochemistry (IHC) studies in human prostate cancer tissues showed a positive correlation between increased mast cell density, MMP9 production confined almost entirely to the tumor-infiltrating mast cells, and well differentiated tumors – supporting the observations observed in the TRAMP mouse studies [114]. Whether mast cell-derived MMP9 plays a role in driving early invasion of human prostate cancer is yet to be elucidated. One caveat of this model is that TRAMP mice are a model of neuroendocrine prostate cancer, while the majority of cases of prostate cancer in humans are adenocarcinoma. Consequently, we specifically wanted to study the role of mast cells in early invasion in models of adenocarcinoma.

One of the pertinent rodent models of precancerous lesions of the prostate is the PhIP rat model, where dietary 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) induces pre-invasive/carcinoma in situ lesions in the ventral prostates of Fischer344 rats [138]. Previous studies have sought to understand the lobal specificity of the model, and Nakai *et al* found an increase in mutations in all lobes of the prostate upon dietary exposure of PhIP, thus suggesting that mutation does not alone explain the development of the precancerous lesions [138]. Nakai *et al* then quantified immune cells in this model, and found that macrophages and mast cells increased in number specifically in the ventral lobes upon PhIP exposure. These results suggested a potential role for inflammatory cells, including mast cells, in promoting precursor lesion development.

We aimed to study whether the increases in mast cells in the ventral lobes of the rats are a transient or a long term effect. To do this, tissues were used from a prior PhIP study performed by Sfanos *et al*. In this study, Fischer344 rats were treated with PhIP (200 ppm in the diet for 20 weeks), prostatic *E. coli* infection (prostatic inoculation at week 10), or both and monitored to an age of 52 weeks [139]. This allowed us the opportunity to quantify mast cells in the ventral prostates of PhIP treated rats who had not received PhIP for 32 weeks, as well as rats with prostatic infection, elucidating whether the mast cell increase was simply in reaction to the presence of PhIP and/or inflammation, or if the increase was a long term effect. This was accomplished via toluidine blue staining and quantification of mast cells in the ventral lobes of the rats in each treatment group. While this study examined whether mast cells are simply reacting to the presence of mutagen in the microenvironment, this study does not actually elucidate a cause and effect relationship between mast cells and tumor invasion. Consequently, we then pursued a mast cell knock-out study in mice that are known to develop invasive prostate cancer as part of their aging process, in order to determine if the presence or absence of mast cells in the prostate has any effect on lesion growth and invasion.

In pursuit of the question of whether the presence of mast cells can affect prostate cancer invasion, we conducted a mouse study where we crossed a well-known prostate cancer mouse model, the HiMyc mouse, with a mast cell knock-out mouse known as the Wsh mouse. The HiMyc mouse is on a BL6 background and over-expresses Myc in an androgen dependent manner in the prostate, developing PIN by 2 weeks and invasive carcinoma in the dorsal lateral prostate by 6 months [140]. The Wsh mouse carries an inversion in the Corin promoter in a FVB background mouse, producing a lack of expression of C-kit, which results

in a mast cell knock-out model [141]. Due to the mixed background nature of this cross, HiMyc-Wsh mice were taken to the F2 generation, to produce littermates of all Myc and Kit genotypes in order to control as much as possible for mixed backgrounds as well as study the dosage effect of Wsh genotype on mast cell number and tumor invasion. In this mouse model, mast cells did not infiltrate into the tumor area. Consequently, this is a model studying the roles of extratumoral mast cells, specifically.

5.3 Methods

PhIP Rat model. Rats were treated with PhIP (200 ppm in the diet for 20 weeks), prostatic *E. coli* infection (prostatic inoculation at week 10), or both and monitored to an age of 52 weeks. Prostates were dissected, formalin fixed, paraffin embedded, and ventral prostates stained with toluidine blue. Slides were scanned with an Aperio ScanScope® CS linear-array scanner (Leica Biosystems). Mast cells were quantified in Aperio for a mast cell number per mm^2 per ventral prostate.

HiMyc-WSH mouse generation. HiMyc mice on an FVB background were crossed with Wsh mice on a BL6 background to the second generation to generate littermates of all genotypes, specifically HiMyc +/-Kit+/+, HiMyc +/-Kit+/-, HiMyc +/-Kit-/-, HiMyc -/-Kit+/-, and HiMyc -/-Kit-/-. All mice were second generation and littermates in order to control for mixed background as much as possible.

Tissue processing. Prostates were dissected and fixed in 10% formalin for a minimum of 48 hours. Tissues were then paraffin embedded and sectioned onto slides.

Slide staining, annotation, and analysis. FFPE slides were H&E stained for annotation for PIN, cribiform PIN, and invasive area by prostate lobe. Adjacent slides were also stained

with toluidine blue (1% toluidine blue in 1% sodium chloride and 7% ethanol) for visualization and quantification of mast cells by lobe. Slides were scanned at 20X using the Aperio ScanScope (CS model, Aperio, Vista, CA) and annotated in Aperio Imagescope. *Statistical analysis*. All statistical comparisons were made using Mann-Whitney test unless otherwise specified.

5.4 Results

Quantification of mast cells in the ventral lobes of PhIP-treated and/or *E.coli* infected rats. Mast cells were quantified in the ventral lobes of the prostates of study animals from all treatment groups (control, PhIP-treated, *E. coli* infected, and PhIP+*E.coli*) (Figure 5.1). PhIP treated mice were found to have significantly higher numbers of mast cells in their ventral lobes compared to control animals ($P<0.005$). *E. coli* infected and PhIP+*E.coli* mice had significantly higher numbers of mast cells compared to the PhIP-treated group ($P<0.05$). All PhIP-treated mice were 32 weeks out from treatment, showing that mast cell increases were a long term effect of PhIP treatment.

Mast cell count by Wsh genotype. Quantification of mast cells showed the expected dosage effect of Wsh phenotype, where mast cell numbers in wild-type mice were significantly higher than heterozygous mice (Figure 5.2). Wsh homozygous mice contained no mast cells.

Mast cell count by prostate lobe. Mast cell numbers were significantly higher in the dorsal-lateral prostates of Wsh heterozygous mice compared to both the anterior ($P=0.0208$) and the ventral prostate ($P<0.0001$, Figure 5.3a). Wsh WT mice also followed the same trend (Figure 5.3b).

Total invasive cancer area percent by genotype. 10 week old mice did not show a significant difference in PIN development by Wsh genotype (data not shown). However, aged (12 month old) Wsh WT mice had significantly higher total invasive cancer area compared to aged Wsh HT mice (P=0.009) (Figure 5.4).

Invasive area percentage by lobe. As is expected with the HiMyc model, all HiMyc mice had significantly greater invasive area in the dorsal-lateral prostate lobes compared to the anterior lobe (P<0.0001) and the ventral lobe (P<0.0001) (Figure 5.5).

Invasive area in dorsal-lateral prostate by Wsh genotype. Wild-type mice had significantly higher total invasive area in the dorsal-lateral prostate compared to heterozygous mice (P=0.01) (Figure 5.6).

5.5 Discussion and Conclusions

In our PhIP rat study, we determined whether mast cell numbers in the ventral prostate are increased in response to PhIP treatment in the long term, specifically 32 weeks post treatment. Our results showed that the increase in mast cells are indeed maintained in the long term in response to PhIP, as well as in response to *E. coli* infection of the prostate (Figure 5.1). The effects of treatment with both PhIP and *E. coli* infection did not appear to be additive (Figure 5.1). These results suggest that the mast cell increase was not just in response to the presence of PhIP or *E. coli* in the microenvironment, as ample time had passed to clear the PhIP and infection. This long term increase in mast cell number may be playing a role in the development of the precancerous lesions in the ventral lobes of the treated rats.

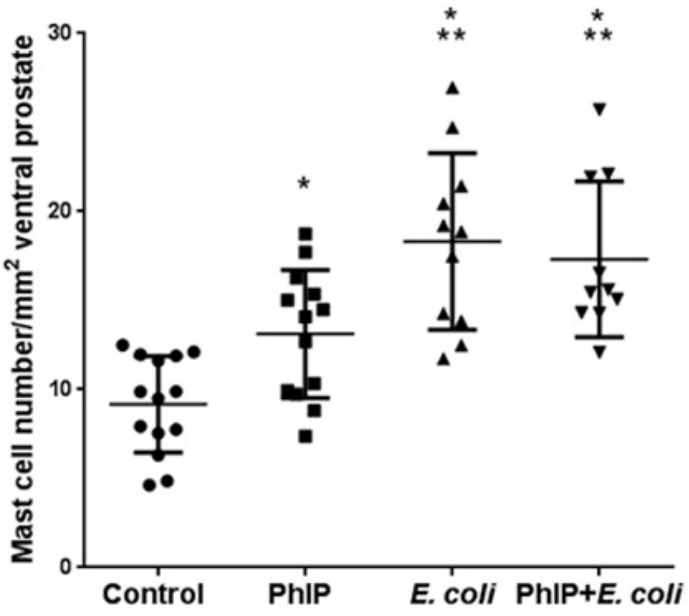


Figure 5.1. Quantification of mast cells in the ventral lobes of study rats. Rats treated with PhIP showed a significant increase in the number of mast cells even 32 weeks post treatment. There was also a significantly higher number of mast cells in rats treated with *E. coli*, and those treated with PhiP and *E. coli*. These results demonstrate that the increase of mast cells in the ventral lobe is a long term effect. * $p < 0.005$ versus control, ** $p < 0.05$ versus PhIP.

In our study in the Himyc-Wsh mice, we found that there was indeed higher invasive area in the dorsal-lateral prostates (DLP) of the mice as is expected with the HiMyc mouse model (Figure 5.5). Interestingly, we also found that the DLP had the highest number of mast cells of all the lobes in the prostates of the HiMyc mice in both Wsh wildtype and Wsh heterozygous mice (Figure 5.3). This suggests that the lobal specificity of invasive caner to the DLP of the HiMyc mouse model may be due to the presence of high numbers of mast cells in the dorsal-lateral lobes of the mice. In addition, total percent invasive area correlated with Wsh genotype, meaning that Wsh+/+ mice, which had no mast cells, had the lowest invasive area and the wildtype mice had the highest (Figure 5.4). This suggests a dosage

effect where higher numbers of mast cells resulted in higher percent invasive area, further evidencing a role for mast cells in early invasion in the HiMyc mouse.

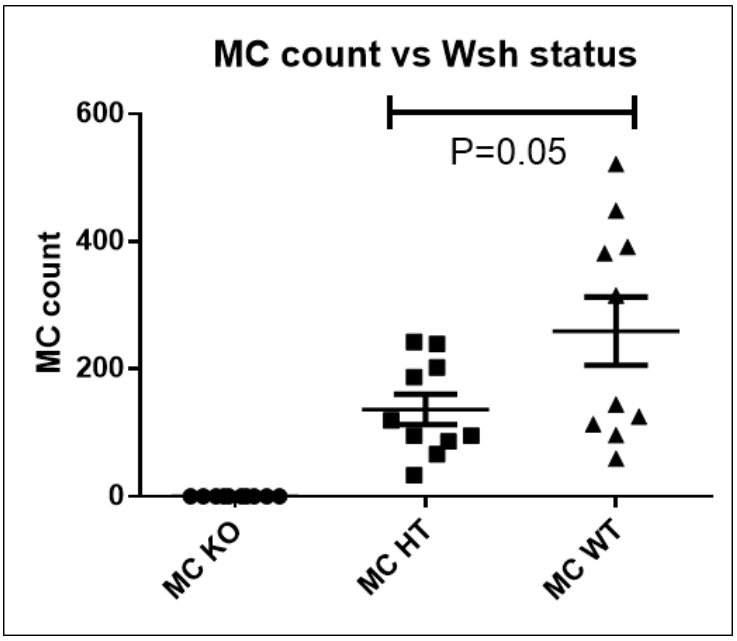


Figure 5.2. Mast cell (MC) quantification. Mice with wild-type Wsh genotype (MC WT) had the highest mast cell count, followed by the heterozygous mice (MC HT), and Wsh homozygous (MC KO) mice had no mast cells.

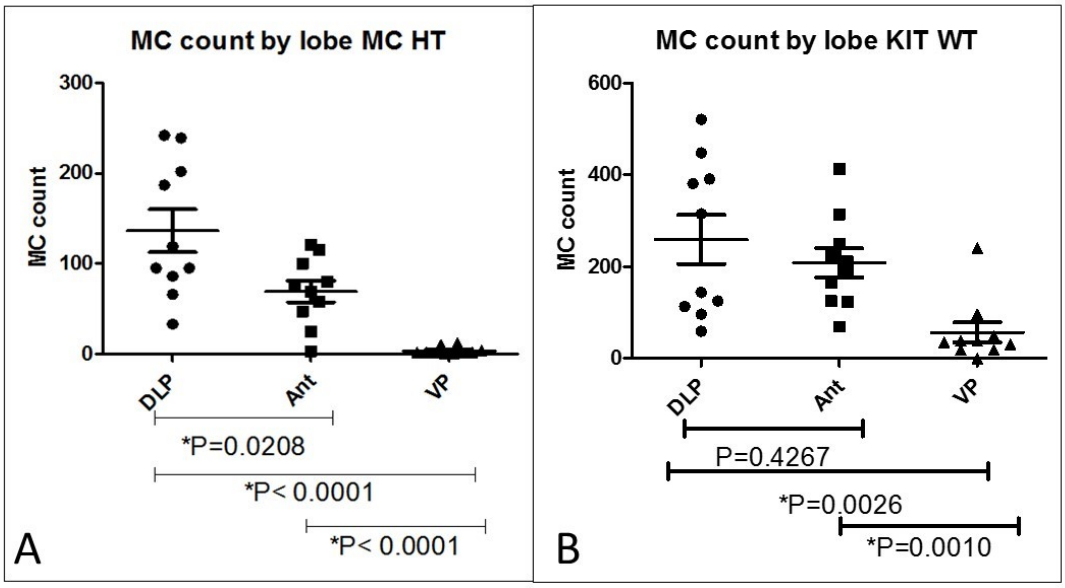


Figure 5.3. Mast cell (MC) number by lobe. In Wsh heterozygous mice, the dorsal-lateral prostate had significantly higher numbers of mast cells compared to both anterior and ventral prostate, while anterior prostate had significantly higher mast cell counts compared to ventral prostate. Wsh wildtype mice (B) showed a similar trend.

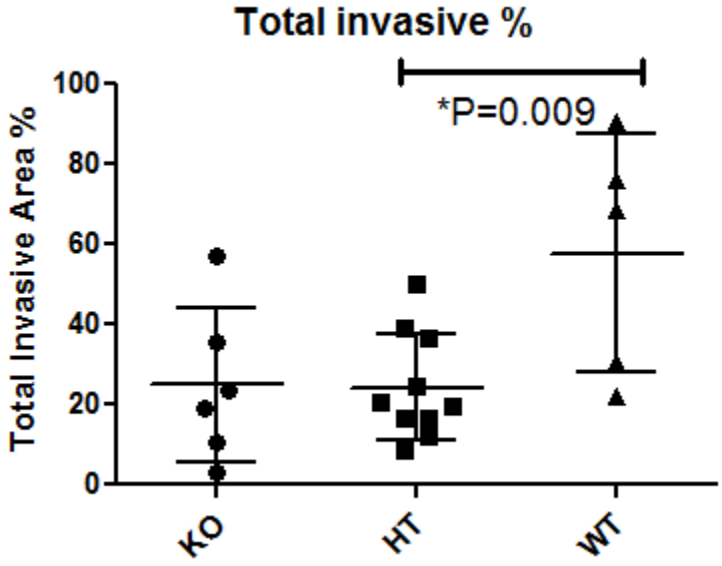


Figure 5.4 Total invasive area percent by genotype. Wild-type mice had significantly higher invasive percentage compared to Wsh heterozygous mice.

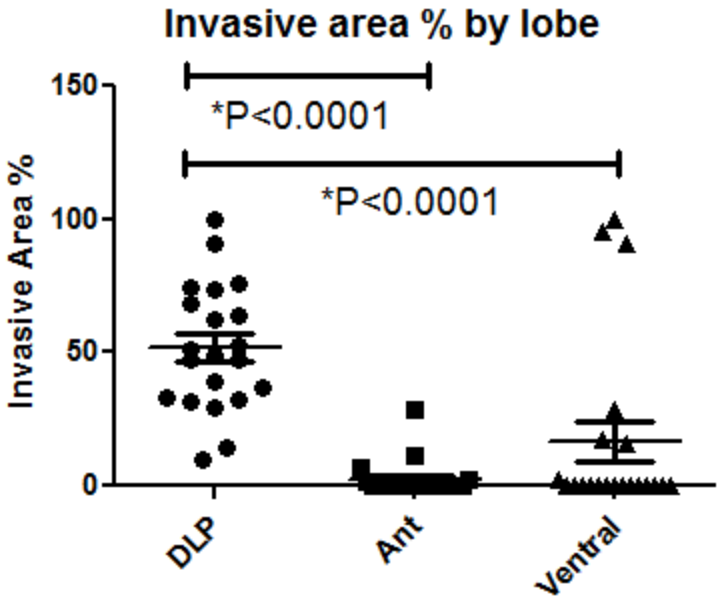


Figure 5.5. Total invasive area percent by prostate lobe. Invasive area percentage was highest in the dorsal-lateral prostate (DLP).

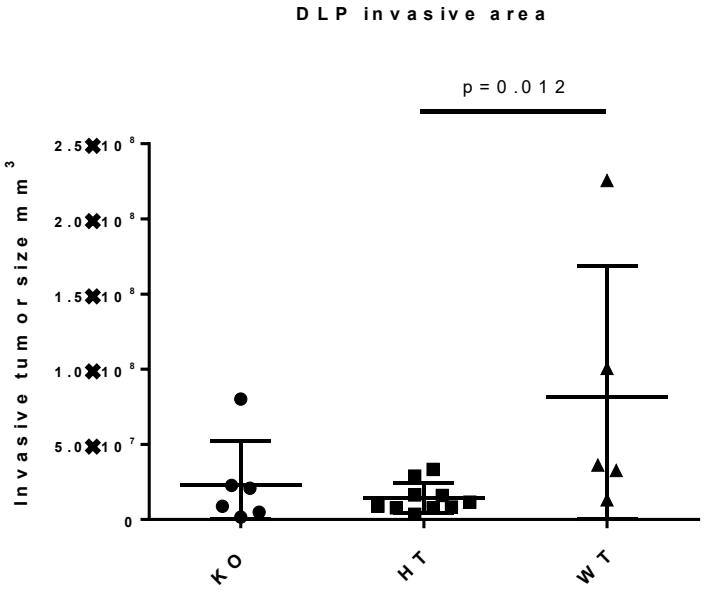


Figure 5.6. Total invasive area in DLP by genotype. Total invasive area was highest in the dorsal-lateral prostate (DLP).

One caveat of the HiMyc-Wsh study was the mixed background of the model, as the HiMyc mouse is on a C57BL6/J background and the Wsh mouse is on a FVB background. Care was taken to keep the cross generations to second generation crosses, as well as produce each genotype as littermates, limiting the number of variables. In addition, there has been discussion over whether pure background mouse studies are indicative of human populations, so it may be appropriate to argue that the mixed background may be more illustrative of effects of mast cells in mixed populations of humans.

VI. CONCLUSION AND PERSPECTIVES

6.1 Mast cells and prostate cancer progression

The relationship between mast cells and prostate cancer has intrigued scientists for some time. Since mast cells are present in the stroma of the healthy prostate and increase in the presence of prostate cancer, the possibility of interactions between mast cells and prostate cancer cells was probable. In addition, as mast cell densities are higher in lower Gleason score tumors than in higher Gleason scores, this relationship did not appear to simply be a linear relationship to the grade of the cancer. Consequently, a number of studies have been conducted to investigate the relationship between mast cells and progression in prostate cancer, with varied results. As was reviewed in Chapter II, Johannson *et al* found high intratumoral mast cell densities to be associated with longer cancer-specific survival [118]. In our study detailed in Chapter II, our findings agreed with Johannson *et al*, with high intratumoral mast cell density being associated with a lower likelihood of PSA progression [120]. In addition, the results of our race disparities study detailed in Chapter III showed that low numbers of intratumoral mast cells were associated with higher risk of metastasis in both

African American and Caucasian American men. Finally, immunostaining of prostate cancer metastases in various rapid autopsy tissues showed no intratumoral mast cells, except in bone (Figure 3.1). As such, our results suggest that mast cells are excluded from highly aggressive prostate cancer with high metastatic potential, and that intratumoral mast cells may either play a protective role or are only allowed into the tumor microenvironment in well differentiated prostate cancer. Given these findings, our next step was to explore whether mast cells contributed to prostate cancer race disparities.

As was reviewed in Chapter III, prostate cancer presents with a significant racial disparity, where African Americans are twice as likely to be diagnosed with prostate cancer compared to Caucasian Americans, and twice as likely to die from their disease. In addition, studies have shown prostate cancer to be more aggressive in African Americans, progressing earlier and faster than Caucasian Americans [87]. Since our results in Chapter II suggest that mast cell density is associated with the potential of prostate cancer to progress or metastasize, we sought to determine if African Americans present at time of surgical intervention with lower intratumoral mast cell densities compared to Caucasian Americans. Our results, showed no significant difference in mast cell densities in higher Gleason score (Gleason $\geq$ 4+3=7) tumors in AA men compared to CA men. AA men did however have lower densities of intratumoral mast cells in lower Gleason score tumors (Gleason $\leq$ 3+4=7), and we hypothesized that AA men with lower grade tumors may tend to do worse. We also stained and analyzed a high risk AA TMA to determine if mast cells have the same relationship with risk of recurrence in AA tissues as was observed in the PSA progression TMA in Chapter II. A similar trend was observed in our data where low intratumoral mast cells were associated with higher risk of PSA progression, however the association did not reach statistical

significance. We did, however, observe a significant relationship between low intratumoral mast cell density and higher rates of metastasis in both CA and AA men, leading us to consider whether the association observed in Chapter II was actually driven by metastasis. Given these data, we conclude that mast cells could be a contributing factor to prostate cancer aggressiveness given their relationship with metastasis and PSA progression in both AA and CA men.

In addition to the inverse relationship between intratumoral mast cells and risk of PSA progression, we also observed a significant relationship between extratumoral mast cells and risk of PSA progression, where high densities of extratumoral mast cells were associated with a higher rate of PSA progression (Table 2.4, Figure 2.2). Given these results, we were interested to study whether mast cells play a direct role in tumor invasion. This was a question best studied in animal models, where cause and effect can be more easily observed. Previous studies had suggested a role for mast cell MMP9 in tumor invasion in better differentiated, or lower grade, tumors in in the TRAMP mouse model [114]. In a study in the PhIP rat model, Nakai *et al* found that mast cells increased in the ventral lobes of treated rats, suggested a role for mast cells in the development of precursor lesions in the ventral lobes of the rats [138]. We quantified mast cells in another PhIP rat study where the rats had been aged another 32 weeks post-PhIP treatment. Results showed that mast cells were significantly higher in the ventral lobes of rats treated with PhIP compared to control rats (Figure 5.1). These results led us to hypothesize that the increase in mast cells in the ventral lobes were not just a reactive inflammatory reaction, and was instead a long term increase that could be interacting with the epithelium in the absence of the PhIP. We also performed a study in mice where mast cell knock-out mice (Wsh mice) were crossed with HiMyc mice, which develop

invasive carcinomas in the dorsal-lateral, and to a lesser extent, the ventral and anterior lobes of the prostate. Our results showed that mast cell knock-out mice developed significantly lower invasive tumor area percentage (Figure 5.4). In addition, all mice developed the greatest invasive area percentage in their dorsal-lateral prostates (Figure 5.5), and the dorsal-lateral prostate also had significantly higher numbers of mast cells than all other lobes (Figure 5.3). Similar to the Nakai study, these results suggest a role for mast cells in the lobal specificity of the rodent model, as well as suggest a role for mast cells in early tumor invasion.

6.2 Mast cell phenotypes: implications of our findings

Mast cells are known to separate into two classical subtypes, specifically MCtc (mast cells that make tryptase and chymase) and MCt (mast cells that make tryptase but not chymase). These subtypes do not appear to be final, however, and studies have shown mast cell subtype ratios to change in disease microenvironments [121]. Mast cells are also known to be diverse in their protease profiles, and studies have shown differences in those profiles as mast cells develop from precursor cells in different tissue microenvironments [1,7]. Given our results that mast cells have a differential relationship with risk of PSA progression depending on whether they are found inside or outside the tumor, we aimed to determine whether mast cells have different phenotypes based on their location in relationship with the tumor. This was accomplished by first analyzing mast cell subtypes in a race disparity TMA set (with the dual purpose of determining if AA tissues present with different mast cell subtype ratios compared to CA tissues). The results of these studies indicated that MCt cells may be proportionately more prevalent in the prostate tumor microenvironment than MCtc cells,

however there were no racial differences in the prevalence of tumor or benign tissue mast cell subtypes observed in our studies.

Next, we used LCM and RNAseq to study mast cell expression profiles in greater detail. Using LCM to microdissect out toluidine blue staining cells, we were able to perform RNAseq to analyze the expression profiles of intratumoral versus benign tissue mast cells and their immediate microenvironments. The RNAseq results indicated significant differences between the collected tumor region and benign region mast cell mRNA. Mast cell related genes, including tryptase subunits, chymase, C-kit, and others, were strongly represented and of roughly equal expression in both tumor and benign, validating that mast cells were indeed enriched for in the samples (Table 4.3). Three genes of interest were chosen for validation and further exploration, specifically C-kit variant 1, CXCR4, and TFE3. RISH and qPCR protocols showed similar patterns as was seen in the RNAseq data, if not in the mast cells themselves, then in their immediate microenvironments and interacting cells. These results confirmed our hypothesis that there are significant differences between mast cells and their immediate microenvironments depending on whether they are found inside or outside the tumor. Our results also suggest that the explanation for our previous data where intratumoral and extratumoral mast cells had an opposite relationship with risk of PSA progression (Figures 2.2, 2.3) lies in the underlying biology of the mast cells themselves, as well as their cellular interactions. This has significant implications for the clinical application of our findings.

While our results for the relationships between mast cells and risk of PSA progression, metastasis, and early invasion are significant, they are not robust enough to currently warrant a direct clinical application. Conversely, our results that biological

differences exist between intra- and extratumoral mast cells suggest that targeting mast cells for therapy in prostate cancer would be a very delicate and potentially dangerous prospect, as intratumoral mast cells appear to be potentially protective against metastasis (or at least excluded from the microenvironments of tumors with metastatic potential), while extratumoral mast cells may actually help drive tumor invasion. However, mast cells may prove a valuable tool as a prognostic indicator, especially in Gleason score 7 prostate cancer, where prognosis is less predictable and definitive treatment is unclear.

In summary, mast cells are of interest in the context of cancer as they are diverse, dynamic, and could be either pro or anti-tumorigenic depending on the context. The experimental content of this thesis supports a role for mast cells in prostate cancer invasion, progression, and metastasis. As such, mast cells are an interesting potential prostate cancer prognostic factor worthy of further study.

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Heidi Anne Hempel, B.S.

Education

2011-PresentPh.D. Candidate, Pathobiology Graduate Program

Johns Hopkins University School of Medicine, Baltimore, MD

2016 Certificate in Health Disparities and Health Inequality

Bloomberg School of Public Health, Baltimore MD

2007-2011 B. S., Major: Cell Biology and Genetics, Minor: Art History,

University of Maryland, College Park, MD

Extramural Research Experience

2007-2010 Summer Fellow,

National Institutes of Health,

National Cancer Institute/Pediatric Oncology Branch Oncogenomics Section,

Advanced Technology Center, Bethesda, MD

Honors and Awards

2016 Johns Hopkins Department of Pathology Young Investigators Award for Excellence in Translational Research

2014 American Association for Cancer Research (AACR) Scholar in Training Award

2011 Inductee, Microbiology Honors Society

2010 Honors Citation, Honors Program

2009-2010 Primannum Honor Society

2009-2010 National Society of Collegiate Scholars

2007-2010 Dean's List

2008-2009 College of Chemical and Life Sciences Certificates for Academic Achievement

2007-2008 Dean's Scholarship

Professional Memberships

2013-present Member, American Association for Cancer Research (AACR)

Teaching Experience

2012-2014 Teaching Assistant and Lecturer, Introduction to Biomedical Sciences,
Johns Hopkins University Bloomberg School of Public Health,
Baltimore, MD

2014 Teaching Assistant and Lecturer, Introduction to Biomedical Sciences Online Course,
Johns Hopkins University Bloomberg School of Public Health,
Baltimore, MD

Publications

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